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Roy Waldo Miner

GROWTH OF PROTOZOA

BY

S. H. Hutner and William Trager (*Conference Chairmen*), H. D. Baernstein, D. L. Baile, F. W. Barber, C. E. Becker, M. Calvin, A. L. Cohen, R. L. Conner, J. Cowperthwaite, J. Filfus, Q. M. Geiman, R. Goldberg, S. Granick, R. P. Hall, L. Hamilton, D. Hendlin, A. Hervey, C. N. Huhtanen, L. Jacobs, W. E. Jacobson, W. H. Johnson, G. Kucker, A. B. Kupferberg, G. Lampson, L. Levy, R. A. Lewin, D. M. Lilly, R. S. Lowy, V. H. Lynch, R. B. McGhee, C. A. Miller, L. Packer, G. E. Palade, B. P. Phillips, I. J. Pintner, L. Provasoli, M. R. R. Rao, C. W. Rees, W. R. Robbins, A. H. Romano, M. Rudzinska, R. Sager, G. R. Seaman, J. G. Shaffer, H. O. Singher, J. C. Sobels, H. Sprince, L. A. Stauber, M. E. Stebbins, C. C. Stock, J. Storm, M. Sussman, R. Sussman, C. B. Troescher, W. J. van Wagtendonk, T. von Brand, M. M. Weber, D. Weinman, M. Williamson, and J. J. Wolken

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Conference Chairmen: S. H. Hutner and William Trager

Consulting Editor: S. H. Hutner

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* This series of papers is the result of a Conference on *Growth of Protozoa* held by the Section of Biology of The New York Academy of Sciences, October 24 and 25, 1953.

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INTRODUCTION TO GROWTH OF PROTOZOA

By R. P. Hall
New York University

Modern progress in the determination of growth requirements has been dependent upon two significant advances in protozoological techniques. The first of these is the establishment of increasing numbers of species in cultures that contain no other microorganisms. This step necessarily involved the development of culture media that would support growth of protozoa through their own biochemical efforts, without the contributory activities of bacteria or other organisms. With one successful result leading to another, new subjects for investigation have appeared in various laboratories. *Peranema*, with an acquired taste for cream, has joined *Euglena* and *Astasia*. Several of the interesting chrysomonad flagellates are thriving in pure cultures, and a few of the dinoflagellates are beginning to respond to patient nursing. *Tetrahymena* now has interesting company in *Paramecium*, and recruits are expected soon from the hypotrichs and Suctoria. The slime-molds and Acrasiales also are beginning to raise problems for several investigators. Finally, some of the more refractory parasitic protozoa seem about to yield to the general techniques that have been successful with many free-living protozoa.

The second technical improvement is the gradual replacement of the old-fashioned broths with carefully measured mixtures of synthetic reagents. Such chemically defined media are leading to the accurate analysis, quantitative as well as qualitative, of growth requirements for more and more species. In several instances, the data have reached such a degree of precision that certain species can be applied to the microbiological assay of particular vitamins and amino acids.

The determination of basic requirements has already paved the way for the analysis of metabolic activities in various free-living protozoa. As a result, fundamental information is being accumulated for several species of flagellates and ciliates. In addition, encouraging progress in the analysis of growth requirements, paced by investigations on *Strigomonas*, promises the early realization of similar aims for a number of parasitic protozoa. Such findings will be extremely important to parasitologists and pathologists in their concern with host–parasite relationships and the practical problems arising from such associations.

For a number of years, occasional papers have marked a steady progress in the study of protozoan growth requirements and have suggested general trends to be expected in future research. The papers collected for the present conference will reveal, however, for the first time, a full-scale composite picture of the future prospects in this field of protozoology. The future is a bright one.

EUGLENA AND VITAMIN B_{12}*

By William J. Robbins, Annette Hervey, and Mary E. Stebbins
The New York Botanical Gardens, Department of Botany,
Columbia University, New York, N. Y.

In previous publications,[14, 15, 16, 17] we have reported observations on vitamin B_{12} and on *Euglena gracilis* var. *bacillaris*. The present paper records further observations on *Euglena* and on its relation to vitamin B_{12}.

Methods

We have used mainly medium I, originally suggested by S. H. Hutner and T. H. Jukes. In addition to mineral salts and thiamine, this medium contains 0.4 per cent L-glutamic acid and 0.4 per cent DL-malic acid. The pH is about 3.0.

Doubling the strength of this medium greatly increased growth, the maximum growth with the 2× solution being approximately twice the maximum with the 1× solution (FIGURE 1).

A systematic examination of the constituents of medium I demonstrated that doubling the concentration of the two organic acids was as effective as doubling the strength of the entire medium. We have therefore modified our medium by adding 0.8 per cent of glutamic acid and 0.8 per cent of malic acid in place of the 0.4 per cent of each previously used.

We have observed also that the condition of the inoculum is important. More satisfactory standard curves were obtained under our conditions if the inoculum was two days old. One-day or three-day old inoculum gave flatter curves and lower maxima.

Viability of Euglena

Under Oil. *Euglena* has remained viable for 19 months in our liquid medium I covered with a layer of mineral oil.[19] The culture was kept in the dark at 15°C. We have kept a liquid culture under oil in continuous light at 25°C. for 15 months.

Vitamin B_{12} in Distilled Water

Variabilities and inconsistencies in some of the work we have attempted led to an examination of distilled water for the presence of vitamin B_{12}. Water, freshly redistilled, and reduced to one-twentieth or one-fiftieth volume under vacuum was negative. Distilled water collected from laboratory wash bottles and concentrated 20 times showed 10 $\mu\mu$g. of B_{12} per ml. (0.5 $\mu\mu$g. per ml. in the original water.) Water from a storage bottle catching distilled water from a Stokes automatic still was found to contain 0.12 $\mu\mu$g. of B_{12} per ml. Freshly distilled water allowed to stand for one month on a laboratory shelf in an open Erlenmeyer flask contained 1.0 $\mu\mu$g. of B_{12} per ml. No macroscopic evidence of growth was observed in any of these samples of water.

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* This investigation was supported in part by the Lucy Benjamin Rogers Fund and The Howard Bayne Research Fund of The New York Botanical Garden.

Freshly distilled water in 500 ml. quantities was placed in 1-liter glass-stoppered bottles and in 1-liter cotton-stoppered flasks. After autoclaving, one set of bottles and flasks was "inoculated" with 0.5 ml. of water from wash bottles containing distilled water. These wash bottles were those in use in the laboratory. After two months at room temperature, the contents of the bottles and flasks were concentrated 50 times under reduced pressure, and the concentrate was assayed for vitamin B_{12}. At this time, there was no macroscopic evidence of growth in any of the flasks or bottles. Even the concentrate showed no turbidity. The entire experiment was run in duplicate. No B_{12} was found in the sterile uninoculated bottles or flasks. The inoculated containers showed the following in μμg. of B_{12} per ml. of original water: Flask 1, 1.1; Flask 2, 2.2; Bottle 1, 0.5; Bottle 2, 0.7. The lower amount of B_{12} in the glass-stoppered bottles resulted probably from the smaller volume of air available.

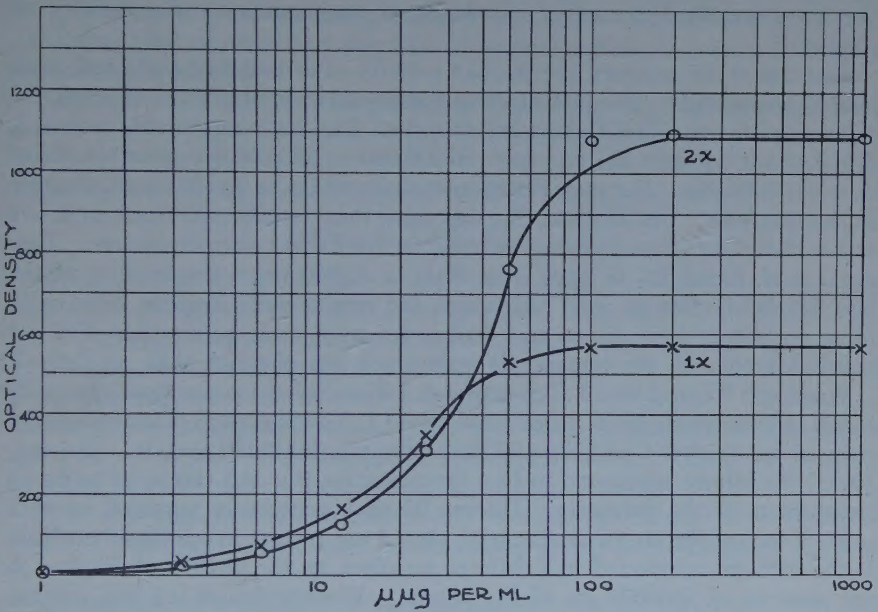

FIGURE 1. Optical density of *Euglena* grown for seven days at 25°C. in continuous light with various amounts of B_{12} in medium I (1X), and in double strength medium I (2X).

We may assume that the B_{12} in distilled water arose from the synthetic activity of microorganisms that developed in the water at the expense of hydrogen, ammonia, methane, or other substances absorbed by the water from the laboratory air. In fact, we isolated from distilled water bacteria and actinomycetes that had the ability to synthesize B_{12}. Such isolations do not, however, prove that those particular organisms were responsible for the results observed.

The formation of vitamin B_{12} in distilled water (or stock mineral nutrient solutions) not kept under sterile conditions may account for the growth of *Euglena* in solutions assumed to be free of B_{12}[18] or be a source of error in the assay of small amounts of the vitamin.

Specificity of Vitamin B_{12} for Euglena

We reported earlier[15] that vitamin B_{12} could not be replaced for *E. gracilis* by vitamin-free casein hydrolysate, carbon-treated peptone, methionine, ascorbic acid, pteroylglutamic acid, a variety of purine and pyrimidine bases, or by xanthopterin and a number of related compounds. Hutner *et al.*[4] found thymidine to be ineffective. Vitamin B_{12a} (hydroxocobalamin) was active.[15] We have examined a number of additional compounds for B_{12} activity on *Euglena.*[17]

Emerson *et al.*[3] reported the B_{12}-like activity of α- and β-ribazole and some related compounds. Samples of these compounds were obtained through the courtesy of Doctor Karl Folkers and tested on *Euglena* in our liquid medium I. For *Euglena,* vitamin B_{12} was from 50,000,000 to 150,000,000 times as active as α- or β-ribazole. For rats, Emerson *et al.* found B_{12} to be 400 times as active as the ribazoles. For *Euglena,* B_{12} was more than 166,000,000 times as active as 1-D-ribityl-5,6-dimethylbenzimidazole or 5,6-dimethylbenzimidazole. Emerson *et al.* found B_{12} to have from 8000 to 20,000 times the activity of the two benzimidazoles on rats. Although the results with *Euglena* agree with those on rats in that the α- and β-ribazoles were more active than the two benzimidazoles, all the compounds were much less effective than on the rat.

Woolley[21, 22] found that 1,2-dimethyl-4,5-diaminobenzene increased the synthesis of vitamin B_{12} by *B. megaterium* while 1,2-dichloro-4,5-diaminobenzene reduced its synthesis and, in sufficient amounts, inhibited growth. He suggested the former compound to be a precursor for B_{12}, and the latter to be an antagonizer of the precursor. Doctor Woolley generously supplied us with some of each of the above compounds At 0.5 mg. per ml. in our liquid medium I, the dichloro compound had little or no effect on the growth of *Euglena* in the presence of 0.00002 μg. of B_{12} per ml. Woolley found 0.1 mg. per ml. under his conditions reduced growth of *Euglena* by 50 per cent. In our experiments, the dimethyl compound had less than 1/100,000 the effect of B_{12}. Woolley reported 1,2-dimethyl-4,5-diaminobenzene to be between 1/500,000 and 1/1,000,000 as effective as B_{12} for *Euglena gracilis.*

Through the courtesy of Doctor T. H. Jukes, a sample of ammonia cobalichrome was obtained from Doctor V. Petrow under his number AC2. This compound is vitamin B_{12} with ammonia substituted for cyanide. Both filtered and pasteurized samples of ammonia cobalichrome had about one-half the activity of vitamin B_{12}. According to Cooley *et al.*[1] ammonia cobalichrome has 2.5 times the activity of B_{12} for *Lactobacillus lactis,* 1.5 times the activity of B_{12} for *Escherichia coli,* and activity equal to B_{12} for the chick.

Pfiffner *et al.*[10] reported the isolation of an organism from bovine rumen that, under anaerobic conditions, produced a mixture of cobalt-containing pigments belonging to the B_{12} group. Two major constituents were obtained in crystalline form. They had the same order of activity on *L. leichmannii* and *L. lactis* Dorner as vitamin B_{12} but were inactive on chick growth and rat antihemorrhagic kidney tests. Doctor Pfiffner kindly supplied us with samples of crystalline pseudovitamin B_{12},[2] crystalline pseudovitamin B_{12b}, and cyano-pigment C. Pseudovitamin B_{12} is about as active on *L. leichmannii* and *L. lactis* as vitamin B_{12}; pseudovitamin B_{12b} has about half the activity of B_{12} on *L. leichmannii*, *L. acidophilus*, and *L. lactis* and cyano-pigment C has only a trace of activity on *L. leichmannii.*[9] Pseudovitamin B_{12} and pseudovitamin B_{12b} had the same order of activity on *Euglena* as vitamin B_{12}. The activity of cyano-pigment was about half that of vitamin B_{12}.

Doctor C. A. Elvehjem furnished us with a preparation of a substance containing cobalt,[6] which was isolated from rat faeces. The material was active on *L. leichmannii* and the chick, but had little or no activity for the rat. In our hands, this material had about twice the activity for *E. gracilis* as that reported for *L. leichmannii.*

Although vitamin B_{12} has a high degree of specificity for *Euglena gracilis* var. *bacillaris*, our results demonstrate that substances exist in nature which have B_{12} activity for *Euglena* and little or none for animals. Assays for B_{12} made with *Euglena* are not sure indications of the presence of vitamin B_{12}.

Absorption of B_{12} by Euglena

When *Euglena* was grown for one week in 25 ml. of liquid medium containing from 25 to 250 $\mu\mu$g. of vitamin B_{12} per ml., from 40 to 70 per cent of the original B_{12} activity was recovered from the *Euglena* by brief boiling in water.

The procedure in these determinations was as follows: At the end of one week, the *Euglena* was removed by centrifugation and washed. The B_{12} was determined in the supernatant and washings. A dry-weight determination was made on an aliquot of the *Euglena.* The balance of the *Euglena* was brought to a boil in water and the extract was assayed for B_{12} (TABLE 1).

Since the *Euglena* made up about one per cent by volume of the liquid in which it was grown, substantial accumulation of B_{12} in the organism had occurred.

We cannot say whether the B_{12} unaccounted for (not recovered from the supernatant medium or from the *Euglena*) was destroyed in the metabolism of the organism, bound in the *Euglena* in such fashion that it was not recovered by boiling, or was the result of inherent errors of the assay. Extractions of the *Euglena* with dilute HCl did not increase the yield of B_{12}.

When *Euglena* was suspended in liquid medium with a large amount of B_{12}, the B_{12} was rapidly absorbed and reached a maximum within 24 hours. The *Euglena* contained about 2500 $\mu\mu$g. B_{12} per mg. dry weight. The experiment was performed as follows:

Washed *Euglena*, equivalent to 177 mg. in dry weight, was suspended in 10 ml. of medium with 0.258 μg. of B_{12} per ml. The *Euglena* and the supernatant were assayed at intervals up to 28 hours (TABLE 2).

Variability of Euglena

Spontaneous "mutations" of *Euglena gracilis* var. *bacillaris* occur.[11] Occasional chlorotic (white) colonies (1 in 25,000–30,000 colonies) appear when our "normal" green form, originally derived from a single cell, is plated. The number of variants, however, is increased by various treatments, including streptomycin, aureomycin, or elevated temperature treatment.

Streptomycin. As first observed by Provasoli, Hutner, and Schatz[13] and confirmed by others,[5, 7, 8] streptomycin (SM) induces the development of stable chlorotic strains of *Euglena*. Grown in our liquid medium I with 50.0 mg. of SM per ml., the culture of *Euglena* became chlorotic. Mass transfers to medium I without SM resulted in a chlorotic culture, which has been maintained by successive transfer[3] at weekly intervals through 105 passages. Occasional platings during these passages, including the 102nd, gave nothing but white colonies; not a single green or yellow was observed.

TABLE 1

ASSAYS OF B_{12} FOR *Euglena* GROWN ONE WEEK IN LIQUID MEDIA WITH VARIOUS AMOUNTS OF B_{12}

	Expt. 1	Expt. 2	Expt. 2	Expt. 2
B_{12} in original medium, μμg. per 100 ml.	10,000	2500	10,000	25,000
B_{12} recovered from supernatant, μμg. per 100 ml.	880	Less than 100	Less than 100	Less than 100
μμg. B_{12} recovered from *Euglena* in 100 ml.	7,000	940	4,200	13,600
Dry wt. *Euglena*, mg. per 100 ml. medium	177	260	300	320
B_{12} in *Euglena*, μμg. per mg. dry wt.	40.0	3.6	14.0	42.5
B_{12} unaccounted for, μμg. per mg. dry wt. *Euglena*	12	5–6	19	35

TABLE 2

ASSAYS OF VITAMIN B_{12} FOR LIVING *Euglena* (177 MG. DRY WEIGHT) SUSPENDED FOR VARIOUS PERIODS OF TIME IN 10 ML. OF MEDIUM CONTAINING 2.58 μG. B_{12}

Time in hours	μg. B_{12} in extract of *Euglena*	μg. B_{12} in supernatant
0.0	0.007	2.58
1.0	0.172	2.57
4.5	0.320	2.67
22.0	0.410	2.18
28.0	0.440	2.04

The action of SM is not an all or none effect. With smaller amounts of SM, various color types were obtained; *i.e.*, on plating, colonies developed that were white, yellow, or pale green. With the larger amounts of SM, all colonies were white.

The effect of the smaller amounts of SM increased with time. Platings made after five days from a culture in liquid medium I containing 2.5 mg. SM per ml. showed 88 per cent of the colonies chlorotic and 12 per cent green; after 13 days' contact with the SM there were 99 per cent chlorotic colonies and 1 per cent green.

Euglena was highly resistant to the inhibitory action of SM. We observed no reduction in number of colonies when *Euglena* was plated in agar medium I with 20 mg. SM per ml.

The action of SM in producing chlorotic *Euglena* is not a selective action, *i.e.*, it does not act by favoring the development of the rare chlorotic individuals that may be present in the stock green culture; the effect is direct on the individual organisms. For example, a three-day-old green culture was plated in agar medium I containing various amounts of SM. The number of colonies was not affected by SM but the type of colony was influenced (TABLE 3).

Different types of green colonies were observed on the plates with 0.5 and 1.0 mg. of SM per ml. Some were pale green, others dark green, and some were green in the center and pale green or white along the edges. The yellow colonies were also of different types. Some were yellow and some yellow with green areas (sectorial or patch mutants?).

Individual variations in resistance to bleaching by SM were noted. Isolations made from dark-green colonies in a plating of an SM-treated culture in which only one per cent of the colonies were green were more resistant to SM than the stock green culture (TABLE 4).

TABLE 3

EFFECT OF STREPTOMYCIN ON NUMBER AND TYPE OF COLONIES OF *Euglena* IN AGAR MEDIUM I

Streptomycin, mg. per ml.	No. colonies per plate	Per cent colonies		
		Green	White	Yellow
0.0	370	100	0	0
0.5	396	91	5	4
1.0	440	15	77	8
2.0	476	0	100	0
3.0	417	0	100	0
4.0	378	0	100	0

By selecting the occasional green colony appearing on plates that contained suitable amounts of SM, a strain was obtained that showed a faint green color in liquid medium when grown with 5 mg. SM per ml. Our stock culture showed no green with 2 mg. SM per ml.

Aureomycin. Aureomycin added in sufficient amounts to our liquid medium I produced cultures of highly motile chlorotic organisms. Mass transfers to media free of aureomycin yielded green cultures, however. On plating cultures treated with aureomycin for 7 days, a number of white colonies were obtained (TABLE 5).

Isolations from the white colonies have been maintained for 15 months through 19 successive transfers. Platings made of the 9th passage showed 100 per cent white colonies.

The development of chlorotic organisms in media containing aureomycin is not the result of selection of the occasional chlorotic individual that may be in the stock culture. This was demonstrated by plating *Euglena* in agar medium I with 5.0, 1.0, or 0.5 mg. aureomycin per ml. No growth occurred with 5.0

mg. aureomycin per ml.; 1.0 or 0.5 mg. per ml. did not affect the number of colonies that developed. All the colonies on the aureomycin plates were chlorotic except for 2 green ones (resistant?),which appeared out of a total of 4460 on the plates with 0.5 mg. aureomycin per ml. It was observed, however, that some of the chlorotic colonies were considerably smaller than the balance. Isolations were made into media free of aureomycin from 12 of the small colonies. Of these, 10 developed as chlorotic cultures, and two as green ones.

Other Toxic Substances. Aside from the bleaching effects, *Euglena* is quite resistant to relatively large amounts of streptomycin or aureomycin under our conditions. A number of other substances were tested for toxicity by dipping filter-paper discs (Schleicher and Schuell, No. 74E, 0.5 inch in diameter) in solutions of the substances and laying them on agar medium I seeded with *Euglena* and supplemented with B_{12}. Expressing the results in μg. per ml. in the original solutions, no inhibition was observed with glucuronolactone at 4000; *p*-aminosalicylic acid, chloromycetin, glucomycin, penicillin G, and penicillin X at 2000; aureomycin at 1280; neomycin and terramycin at 1000; viomycin at 500; subtilin at 250; illudin M at 200; bacitracin, enniatin A, 2-methyl-3-hydroxy-1,4-naphthoquinone, and D-usnic acid at 125; aspergillic acid, gramicidin, 2-hydroxy-1,4-napthoquinone, pleurotin, polymyxin, spinulosin, and tyrocidine at 62.5; polyporic acid at 31.

TABLE 4

THE PROPORTION OF GREEN AND WHITE COLONIES FOUND ON PLATING STOCK GREEN *Euglena* AND A RESISTANT ISOLATE IN MEDIA CONTAINING VARIOUS AMOUNTS OF STREPTOMYCIN

Streptomycin, mg./ml.	Stock green, per cent		Isolation No. 88, per cent	
	White	Green	White	Green
0.0	0.0	100.0	0.0	100.0
1.0	30.0	70.0	4.0	96.0
2.0	99.9	0.1	99.0	1.0

TABLE 5

BLEACHING EFFECT OF AUREOMYCIN

Aureomycin mg./ml	White colonies per 1000
0.0	0
1.25	30
5.0	120

Borrelidin evidenced inhibition under the filter-paper disc at 0.5 μg. per ml. and produced inhibition zones 44 mm. in diameter at 5 μg., and 70 mm. at 500 μg. per ml. Mycophenolic acid at 125 μg. per ml. caused a zone of partial inhibition 40 mm. in diameter; actinomycin at 62.5 μg. per ml. an inhibition zone 24 mm. in diameter; bis-(2-hydroxy-5-chlorophenyl)-methane at 10,000 μg. per ml. an inhibition zone 16 mm. in diameter; 2-methoxy-1,4-naphthoquinone at

62.5 μg. per ml. an inhibition zone of 16.5 mm. With 2-hydroxy-1,4-naphthoquinone at 1000 μg. per ml., patulin and 2-amino-1,4-naphthoquinone at 125 μg. per ml., and nigericin and 2-methylmercapto-naphthoquinone at 62.5 μg. per ml., inhibition was observed under the filter-paper disc but not beyond its limits.

Those substances, which showed some toxicity with the filter paper discs, were each used in a series of dilutions in liquid medium I. Observations were made macroscopically and microscopically after 3 days' and 8 days' incubation. In this acid medium (pH 3.2), borrelidin was the most toxic to *E. gracilis* (TABLE 6) and 2-amino-1,4-naphthoquinone, nigericin, and patulin the least toxic.

Botulinal toxin type A (P3-P5), obtained through the courtesy of Dr. Carl Lamanna, had no inhibitory effect on *Euglena* at 2.5 mg. per ml. in our medium I containing 50 μμg. B_{12} per ml. The toxin was dissolved in 0.43 per cent sodium chloride and filtered sterile.

TABLE 6

MINIMUM AMOUNTS (μg./ml.) OF VARIOUS SUBSTANCES THAT INHIBIT *E. gracilis* IN MEDIUM I

	3 days		8 days	
	Complete	Partial	Complete	Partial
Borrelidin	0.3	0.07	0.6	0.07
Mycophenolic acid	0.5	0.13	2.0	0.13
2-methoxy-1,4-naphthoquinone	1.0	0.5	2.0	1.0
2-methylmercapto-1,4-naphthoquinone	2.0	1.0	4.0	1.0
2-hydroxy-1,4-naphthoquinone	4.0	—	8.0	2.0
bis-(2-hydroxy-5-chlorophenyl)methane	8.0	4.0	16.0	4.0
2-amino-1,4-naphthoquinone	8.0	0.5	16.0	4.0
Nigericin	16.0	2.0	16.0	2.0
Patulin	16.0	4.0	16.0	4.0

Temperature. Higher temperatures induce variants of *Euglena.* Exposure of our stock green culture to 36°C. in the dark for five days resulted in 73 per cent chlorotic (white) and 27 per cent green colonies; for 14 days, 87 per cent white and 13 per cent green; after 22 days, all the colonies were white. A mass transfer from this chlorotic culture has been maintained through 59 weekly transfers in liquid medium. The effect of 22 days' exposure to 36°C. in the dark was similar to that obtained with sufficient amounts of streptomycin.

Exposure to 30–32°C. for 19 days gave us only 0.2 per cent white colonies.

Short treatments at 36°C. (6 hr.) or at 30–32°C. (5 days) gave a few yellow colonies and a few white. Exposure to 40°C. for periods ranging from 15 minutes to 6 hours or to 45°C. for 15 minutes to 2 hours did not show bleaching of the surviving organisms. Some irregularities in survival were noted with the treatments at 40°C. and 45°C., which suggested variation between individuals in susceptibility to these high temperatures.

The bleaching effect did not appear to be caused by temperature *per se.* It seemed that the temperature treatment had to be such that growth of the organism occurred. Our results on the effect of higher temperatures on the bleaching of *Euglena* are similar to those obtained by Pringsheim.[12]

Strains of Euglena

Isolations from Colonies: In the course of our experiments in which *Euglena* was plated, a number of color variants were noted and isolated. The colonies from which the isolations were made were various shades of green, white to cream color, and various shades of yellow to ocher. These isolates (over 100) were maintained in liquid culture, and some of them were plated from time to time to determine the composition of the populations. They varied not only in color but in rate of growth, in stability, and in resistance to unfavorable factors. All of them required B_{12} for growth.

The instability of some of these variants interested us because, as far as is known, *Euglena* reproduces only by fission, no sexual stage having been demonstrated. Many of the white isolates remained stable for 36 successive passages made at intervals of three weeks. They differed in vigor of growth and in whiteness, some being cream colored. Some isolations from colonies that appeared white grew as white cultures for a few transfers but, eventually, with continued transfer, they became pale green. On plating cultures of this type, white, green, and a few yellow colonies were obtained. Isolations, including 14 single-cell isolates, which grew in liquid media as dark-green cultures, differed in vigor of growth, size of individual organisms, amount of green pigment in an organism, and resistance to streptomycin. Other differences could probably have been found.

Three types of pale-green cultures were isolated from colonies. We had eight isolates that produced light-green cultures when first isolated but which, on successive transfers, became dark green. There were three additional isolations that, on successive transfers, became paler, and one that remained pale green through 28 successive transfers made at intervals of three weeks.

Yellow isolates were especially unstable. Many transfers to liquid media were made from yellow colonies. While the original culture from a colony was yellowish and showed a high proportion of yellow colonies on plating, there were always some white and some green colonies present. On successive transfers, the cultures of some yellow isolates became green and showed a predominance (nearly 100 per cent) of green colonies on plating. Others became white and showed few if any green or yellow colonies on plating. Repeated plating and isolation from yellow colonies gave us in no instance a stable-yellow culture.

Our results may be illustrated, in part, by the history of a single isolation, no. 35. This isolation was made from a white colony, in a plating of a pale-green culture. It grew in liquid media as a white culture but, on continued transfer, it became pale green and has remained in that condition up to date (31st transfer). Platings of early transfers showed a predominance of white with some green and some yellow colonies, while platings of later transfers gave a higher percentage of green colonies. From the plating of the eighth transfer of isolation no. 35, an isolation (no. 70) was made from a yellow colony, one (no. 72) from a pale-green colony, and two (nos.71 and 73) from white colonies. Isolations nos. 71 and 73 have given 100 per cent white colonies through 23

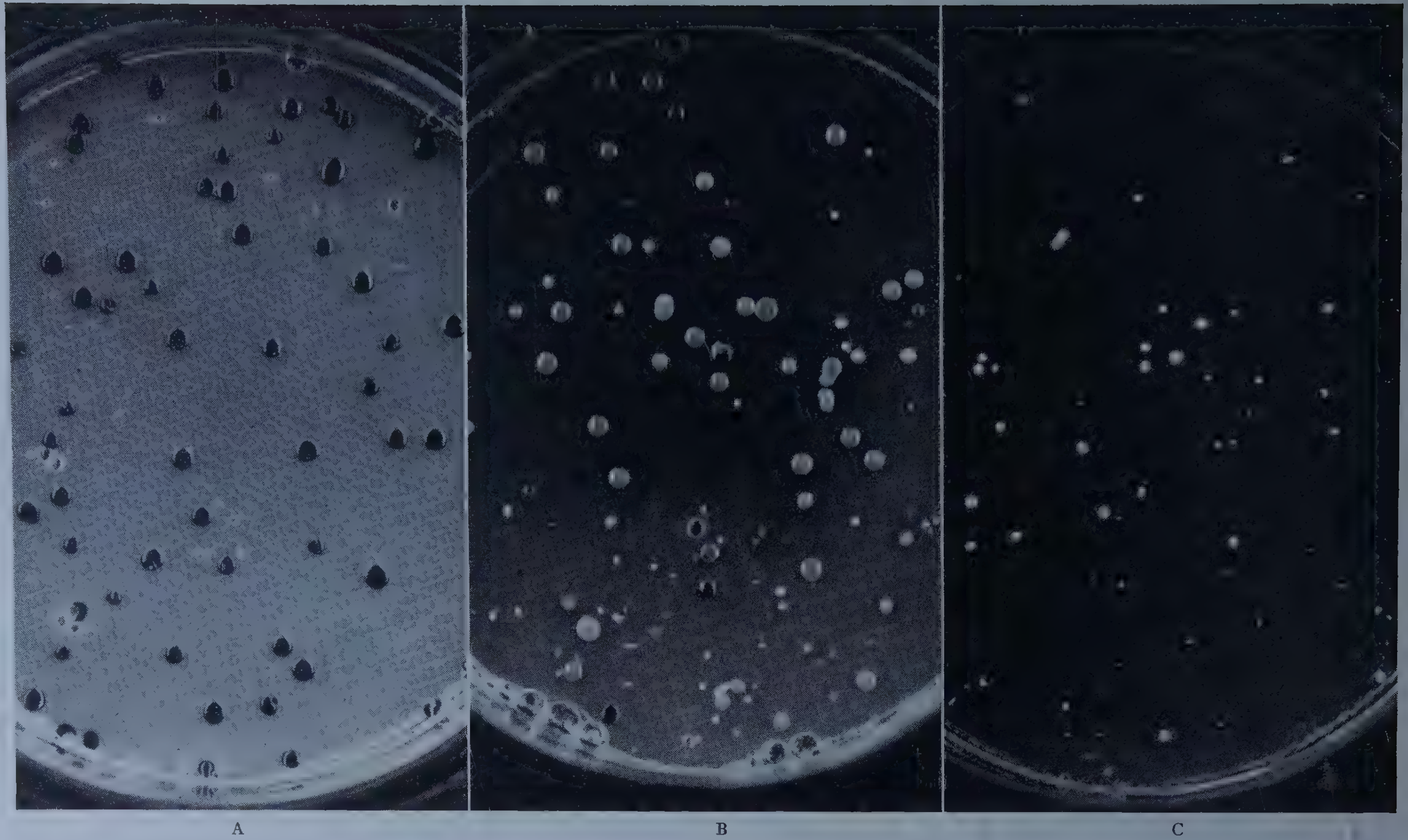

FIGURE 2. Platings of (A), stock green culture; all colonies green; air bubbles in agar should not be confused with colonies. (B), yellow isolate #70 in 5th transfer; mostly yellow colonies, few green, yellow with green areas or white. (C), white isolate in 15th transfer; all colonies white. Note smaller size of colonies in C. Plates 12 days old.

transfers. Isolation no. 72, which originally showed a majority of pale-green colonies with a few white and a few yellow ones, had become almost entirely white by the 15th transfer. Plating of the original transfer of isolation no. 70, after 13 days' growth in liquid media, showed 97 per cent yellow colonies, with a few green and a few white ones. With continued transfer, the proportion of yellow colonies decreased and that of the green and the white colonies increased. Isolations were made from six yellow colonies in a plating of the 16th transfer of isolation no. 70. These have grown as yellowish cultures and platings have given us green, yellow, and white colonies (FIGURE 2).

Single-Cell Isolates. Even single-cell isolations from a yellow strain were not stable. For example, eleven single-cell isolations were made from a culture (no. 70) derived from a yellow colony. Platings of nine of these, after 11 days' growth in our liquid medium, showed 97–99 per cent yellow colonies. There were from 0.2 to 2.5 per cent white colonies, however, and from 0.03 to 0.36 per cent of the yellow colonies became green after 9–14 days. On serial transfers (18 or 19) in the liquid medium, the proportion of yellow colonies found on plating decreased to a fraction of a per cent in cultures of some of the isolates and completely disappeared in others. Two of the isolates, after 19 transfers, showed nothing but white colonies; three, after 5 to 18 passages, had 90–99 per cent white; one, after 18 passages, had 46 per cent white, 53 per cent green, and 1 per cent yellow; one, in the 12th passage, had 29 per cent white, 70.5 per cent green, and 0.5 per cent yellow and, in the 18th passage, 83.9 per cent white, 16 per cent green, and 0.1 per cent yellow. Ternetz[20] described intermediate forms of *E. gracilis*, one of which was yellow (ocher colored) in mass. Single cell isolates of the intermediate forms gave rise to green, yellow, and hyaline organisms. Her experience with yellow forms was much the same as ours. Ternetz was unable to define the conditions for obtaining yellow cultures and found them only once. We have been able to induce their formation by suitable treatments with streptomycin or elevated temperatures.

Our experience indicates that stock cultures of *Euglena* suitable for the assay of B_{12} can be maintained without difficulty in medium I at 25°C. in continuous light. There are conditions (for example, elevated temperatures, presence of streptomycin or aureomycin) that induce variation in the organism. How the variation occurs and why, even under "normal" conditions, some isolations are unstable, including single-cell isolates, is a problem that remains to be answered. Two factors are important in the maintenance of *Euglena* cultures. One is the variability of the organism, and the other is competition between different types. We prepared a mixture consisting of 80 per cent of a stock green and 20 per cent of a white form. After seven subcultures, we found 100 per cent green and no white. For these isolations the green form outgrew the white. In other cultures, the whites appeared to outgrow the greens. In general, cultures of *Euglena* derived from colony or single-cell isolations and grown under our conditions were populations rather than clones.

Summary

Raising the concentration of L-glutamic acid and of DL-malic acid from 0.4 per cent to 0.8 per cent in our medium I approximately doubled the growth of

Euglena with the larger amounts of vitamin B_{12}. Distilled water that had stood under nonsterile conditions contained up to 2.0 $\mu\mu$g. of B_{12} per ml. Freshly distilled water or the same kept under sterile conditions was free of B_{12}. *Euglena* was maintained in liquid culture under a layer of mineral oil for 19 months at 15°C. in the dark. Vitamin B_{12} was from 50 to 150 million times as active for *Euglena* as α- or β-ribazole and more than 166 million times as active as two benzimidazoles previously reported to have some activity on the rat. Dichloro-4,5-diaminobenzene was ineffective on *Euglena* at 0.5 mg. per ml., and 1,2-dimethyl-4,5-diaminobenzene had less than $1/_{100,000}$ the effect of B_{12}. Pfiffner's pseudovitamin B_{12} and pseudovitamin B_{12b} had the same order of activity on *Euglena* as vitamin B_{12}. Cyano-pigment C had about one-half the activity of B_{12}. A cobalt-containing material isolated by Elvehjem and inactive on the rat but active on *L. leichmannii* and the chick was active for *Euglena*. Vitamin B_{12} was rapidly absorbed by *Euglena* and accumulated in the organism from dilute solutions.

Of the number of substances tested, borrelidin was the most toxic for *Euglena* in our medium. A preparation of botulinal toxin had little or no effect even at 2.5 mg. per ml. Streptomycin in sufficient amounts produced permanently bleached (chlorotic) strains of *Euglena*. Intermediate amounts of streptomycin caused the development of pale-green and yellow (ocher) strains. Chlorotic strains of *Euglena* were obtained also with aureomycin and by growing *Euglena* at 36°C. for several days in the dark. Permanent and complete bleaching of a green culture resulted from 36°C. for 22 days in the dark. Pale-green and yellow variants were obtained by short temperature treatments. Over 100 strains of *Euglena* were isolated. They differed in color, vigor of growth, resistance to streptomycin, stability, and in other ways. All required vitamin B_{12}. Many strains of chlorotic *Euglena* appeared to be stable on continued transfer. Many strains were unstable. This was especially marked for the yellow isolates. Even single-celled yellow isolates produced green and chlorotic organisms as cultivation was continued. Cultures of *Euglena* derived from colony or single-cell isolations and grown under our conditions were populations rather than clones.

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Discussion of the Paper

B. S. Schweigert, *American Meat Institute Foundation and Department of Biochemistry, University of Chicago:* We have found that *Euglena gracilis* var. *bacillaris* is of great value for synthesizing compounds that are randomly labelled with C^{14} when grown in the presence of $C^{14}O_2$. In experiments conducted in collaboration with Irwin A. Rose and Mancourt Downing, randomly labelled ribonucleosides and desoxyribonucleosides have been prepared. Studies on the incorporation of the base as well as the sugar moieties of the ribosides have been conducted with a number of organisms. Since $C^{14}O_2$ was the only carbon source utilized by *E. gracilis* under our conditions, all compounds are uniformly labelled. *E. gracilis* is particularly valuable for this work, since it contains rather high amounts of nucleic acids (3–4 per cent ribonucleic acid and 0.8 per cent desoxyribonucleic acid). We believe that other important compounds that are difficult to synthesize and label uniformly (amino acids, constituents of the lipid and carbohydrate fraction, *etc.*) could be readily isolated from *E. gracilis* grown in the presence of $C^{14}O_2$. A preliminary report of our studies has been published (1952. Federation Proc. **11:** 276).

NUTRITIONAL STUDIES WITH *CHLAMYDOMONAS REINHARDI*

By Ruth Sager* and S. Granick
The Rockefeller Institute for Medical Research, New York, N. Y.

At present, we are investigating the genetic mechanisms involved in chlorophyll biosynthesis and chloroplast development. For this project, it has been necessary to use a unicellular organism which is photosynthetic but can also be grown in the dark, and which has a sexual phase in its life-cycle. The ability of the organism to grow in the dark has been of particular importance because of our interest in mutants unable to synthesize chlorophyll and which are therefore non-photosynthetic. The sexual requirement is necessary for carrying out the genetic analysis. The organism chosen, *Chlamydomonas reinhardi*, a unicellular green alga, was isolated by Professor G. M. Smith, to whom we are indebted for the mating type strains used in this work.

The nutritional studies to be reported here are concerned, first, with vegetative growth of the organism in light and dark and, second, with the nutritional conditions which we have found to be of critical importance in controlling the sexual phases of the life-cycle.

1. *Nutrition of Vegetative Cells*

Algae are reputedly slow growing organisms which have been difficult to culture in the laboratory. Currently, however, improved methods for algal culture are being developed. The present status of algal nutrition and physiology is discussed in two comprehensive recent reviews by Myers[1] and Hutner and Provasoli.[2] As yet, there are few quantitative studies of algal growth rates to be found in the literature.

A. *Selection of growth media.* Two types of media have been devised for vegetative growth of *C. reinhardi.* Medium I is designed for photosynthetic growth in the light, and contains no exogenous source of carbon. Medium II contains acetate as carbon source and supports non-photosynthetic growth in the dark. The components of these media are shown in TABLE 1.

Medium I is based upon Beijerinck's four-salt solution which contains ammonium nitrate, magnesium sulphate, monobasic potassium phosphate and calcium chloride.[3] This basic medium has frequently been used for growth of various green algae including *Chlamydomonas*.[4]

Ammonium nitrate is used at a concentration of 0.03 per cent. It has been found that a higher concentration does not enhance growth but lower amounts (0.01 and 0.005 per cent) decrease both the density of growth and the chlorophyll content of the cells. Ammonium and nitrate ions separately can serve as sole nitrogen sources for growth, as can both urea and glutamine. Sodium glutamate, however, is a very poor nitrogen source, as is glycine, both compounds supporting growth for only a few cell generations.

The initial pH of medium I is 6.8, and it remains more or less constant during growth. The initial pH of medium II is 6.2 and it rises as the acetate is utilized. The organism will grow in these media with initial pH adjusted anywhere between pH 5.5 and 8.0, but not at pH 4.0, 4.5 or 5.0. After growth has ceased on a poorly buffered acetate medium in the light, the pH may be as high as 8.8.

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* This work was begun during the tenure of a Merck Fellowship in the Natural Sciences of the Natural Research Council.

A number of elements known to be required by other organisms in trace amounts have been added to this medium. Since the trace element requirements of *Chlamydomonas* are unknown, a suitable mixture could only be determined empirically. Several trace metal mixtures suggested by Hutner and used in the growth of related organisms were tested, and the one giving best growth was adopted. The trace elements except iron are supplied from a trace metal stock solution at high enough concentrations to support heavy growth, but initial toxicity is avoided by the addition of sodium citrate as chelating agent.[2] Iron is added separately as ferric chloride. Since this salt, on standing, gradually hydrolyzes to the insoluble ferric hydroxide, it has been found necessary to make a fresh one per cent stock solution about once a month. In the preparation of these media, if citrate is added first, the precipitation of any salts is avoided. The final medium, even after autoclaving, shows no visible precipitate. We have shown that sodium citrate is itself not utilized by *C. reinhardi* as a carbon source for growth. Versene (ethylenediamine tetraacetic acid), another recommended chelating agent, was found to be toxic.

TABLE 1
NUTRIENT MEDIA FOR *Chlamydomonas Reinhardi*

	Medium I pH = 6.8		Medium II pH = 6.2	
	g/1	M	g/1	M
Sodium acetate·$3H_2O$			2.0	.015
$NaH_2PO_4 \cdot H_2O$			3.67	.026
K_2HPO_4	0.1	.00057	1.15	.0066
KH_2PO_4	0.1	.00074		
NH_4NO_3	0.3	.0037	0.3	.0037
$MgSO_4 \cdot 7H_2O$	0.3	.0012	0.3	.0012
$CaCl_2$	0.04	.00036	0.04	.00036
$FeCl_3 \cdot 6H_2O$	0.01	.00037	0.01	.00037
Sodium citrate·$2H_2O$	0.5	.0017	0.5	.0017
Trace metal solution	10 ml.		10 ml.	

Trace metal stock solution

H_3BO_3	100 mg/l.
$ZnSO_4 \cdot 7H_2O$	100
$MnSO_4 \cdot 4H_2O$	40
$CoCl_2 \cdot 6H_2O$	20
$Na_2MoO_4 \cdot 2H_2O$	20
$CuSO_4$	4

Medium I, in addition to its use for photosynthetic growth of the organism in the light, is excellent for maintenance of stock cultures. Cells have remained viable for a year or agar slants containing this medium, when kept in plastic-capped test tubes at 15°C.

For growth in the dark, sodium acetate is used as carbon source. On a poorly buffered medium, the pH increases as the acetate is utilized during growth. For example, on medium I, plus 0.1 per cent acetate in the dark, growth increases until the pH reaches 8.4, corresponding to utilization of about 85 per cent of the acetate. In attempts to obtain denser growth in the dark, it has been found possible to supply a higher acetate concentration in a more strongly buffered medium.

Comparing several acetate and phosphate concentrations, the best growth was obtained with 0.2 per cent acetate and $M/30$ phosphate buffer at pH 6.2, where there is enough primary phosphate present to keep the final pH of the culture filtrate below 8.0. Final growth is about twice as heavy with 0.2 per cent acetate as with 0.1 per cent. By adding more acetate when growth has ceased, it has been possible to obtain another doubling of the population, but such a procedure is impractical for routine purposes.

In attempts to improve growth in the dark, a number of compounds have been tested as carbon sources for this organism. In addition, the compounds italicized in the following list were also tested with addition of 0.01 per cent acetate in order to test whether acetate might catalyze the utilization of other carbon sources. Among the compounds tested were: *glucose*, *galactose*, sucrose, lactose, maltose, mannose, *D-xylose*, *L-arabinose*, *ribose*, starch, *ethanol*, glycerol, mannitol, formate, glycerophosphate, propionate, butyrate, formaldehyde (10^{-4} M), oxalate, tartrate, pyruvate, malate, fumarate, succinate, α-ketoglutarate, *citrate*, trans-aconitate, *glutamine*, *glutamate*, *asparagine*, *aspartate*, and *glycine*. These compounds were all tested at a concentration of 0.01 M and at several other concentrations. They did not support growth in the dark, with or without added acetate, but were non-toxic in the light. In some instances, vitamin B_1 and yeast extract were added with no apparent effect. A small amount of dark growth occurred with acetylmethyl carbinol, monacetin, and acetylmethyl acetate, but much less than with sodium acetate.

B. *Methods used in selection of the media.* In selecting the components of the medium and studying their effects upon growth, the following methods were employed. Cells were grown in a constant-temperature room at 25°C. with light from "daylight" fluorescent lamps at an intensity of about 600–700 foot candles. Growth experiments were carried out with a roller-tube apparatus similar to the kind employed for growth of tissue cultures. Cell suspensions rotate on the apparatus in cotton-plugged test tubes with a gentle motion, which facilitates gas exchange and maintains a uniform suspension. Under these conditions, healthy cells do not stick to the glass or clump together. To avoid impurities such as fatty acids derived from autoclaving of cotton plugs, test tubes provided with aluminum caps were first sterilized and then plugged with cotton plugs which had been autoclaved separately.

Growth has been measured in terms of increased optical density using a Beckmann spectrophotometer with a test tube adapter. Measurements are made at 750 mμ, thus using the instrument as a turbidimeter. In this way, one avoids measuring growth as a function of chlorophyll concentration. It has been found that optical density varies linearly with cell concentration over the range 2×10^5 to 1×10^7 cells/ml, as checked by cell counts with a haemocytometer.

The data on growth rates presented in this paper have all been determined using the strain *21 gr.* Comparative studies of four strains tested have shown no significant differences in growth rates or final yields.

The data presented in FIGURES 1 and 2 have been plotted as logarithms to the base 2 of the cell concentration following the recommendation of Monod.[5] Since the growth of a microbial population is a process of doubling, the linear slope of the growth curve on a log. plot demonstrates that the population is doubling at a regular rate. On a $\log_2$ plot, the specific rate of doubling, or generation time, g, can be determined directly from the graph, since a unit increase on the Y-axis (*e.g.*, in FIGURE 1, from 18 to 19 on the Y-axis) corresponds to one doubling of the cell population, and the time required for this to occur can be read directly on the X-axis. The generation time g may be read from the curve in this way, or calculated from the equation:

$$g = \log_{10}{}^{2} \frac{(t_2 - t_1)}{\log_{10}b_2 - \log_{10}b_1} = \frac{t_2 - t_1}{\log_2 b_2 - \log_2 b_1}$$

where b_2 is the final concentration and b_1 is the initial concentration of cells during the time interval $t_2 - t_1$.[6]

In FIGURE 1, the growth rates of *C. reinhardi* have been compared under three conditions: medium I in the light, curve B; medium II in the light, curve A; and medium II in the dark, curve C. It was found that in medium I (no acetate) the growth rate, g, in the light was 9.5 hours per division. It remained constant up to a cell concentration of 1×10^6 and then decreased, the cells reaching final concentration of $8–10 \times 10^6$ cells/ml. The growth rate in the light on medium II (with acetate), shown in curve A, was 7 hours per division in the log phase of growth and reached a final concentration of about 2×10^7 cells/ml. In a number of separate experiments, individual values of g have been obtained ranging from 6.6 to 7.3 hours per division.

These values compare favorably with the growth rates for various algae compiled by Myers.[1] Calculated from his data, the lowest generation times reported are 8.6 hours for *Chlorella* and 8.3 hours for *Scenedesmus*.

Comparison of curves A and B demonstrates that growth in the light is enhanced, both in rate and in final density, by addition of acetate. Myers[1] states that, under conditions of light and carbon dioxide saturation, no case has yet been found in which an organic carbon source accelerated growth. Since our studies were carried out in air, it is possible that CO_2 is limiting growth, although, in that event, one might expect that the final cell concentration on medium I would eventually reach that attained with acetate, but this does not occur.

In the dark, the growth rate on medium II has averaged about 18 hours per division, and the density of final growth is about 3×10^6 cells/ml. as shown in FIGURE 1, curve C. This medium is somewhat toxic initially to cells grown in the dark. With medium I, plus 0.1 per cent acetate, growth rates of 14 hours per division in the dark have been obtained. However, final growth with 0.1 per cent acetate is only about half of that with 0.2 per cent acetate in medium II.

Growth rates in light and dark have also been determined in media supplemented with each of the following components: 0.1 per cent yeast extract; 0.05 per cent enzymatic casein hydrolysate; yeast extract plus casein hydrolysate; 0.1 per cent glutamine; 0.1 per cent glutamate; 0.1 per cent glycine; and 0.1 per cent urea. In no case, has growth rate or final density been enhanced by any of these supplements over that on acetate.

The low rates in the dark indicate that other factors besides acetate are limiting dark-growth. This is borne out by the observation that starch accumulates in the cells during growth on acetate in the dark. The fact that starch accumulates indicates that there is a sufficient store of carbohydrate for cell multiplication. Yet both the rate and final density of growth are considerably lower than those of light-grown cells. There is apparently some imbalance in carbohydrate metabolism by these dark-grown cells but at present there is no evidence of where the difficulty lies.

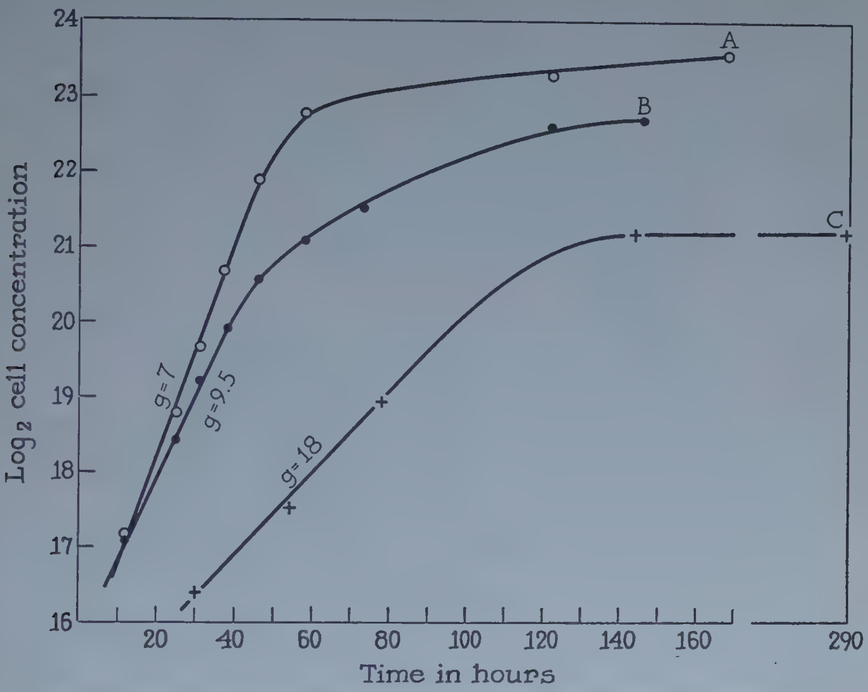

FIGURE 1. Growth of *Chlamydomonas reinhardi* in light and dark. Curve A represents cells grown in medium II (*i.e.* with acetate) in light. The generation time, *g*, is 7 hours in the log phase of growth. Curve B represents cells grown in medium I (no acetate) in light. Here, *g* is 9.5 hours in the log phase of growth. Curve C represents cells grown in medium II in the dark. Here, *g* is 18 hours in the log phase of growth. Inoculum used contained 10^4 cells/ml. grown in medium II in light, in the log phase of growth.

In a number of experiments, it was noted that glutamine could serve as sole nitrogen source for growth in either light or dark, but not as carbon source. In the following experiment, it is shown that the presence of glutamine does not accelerate growth in the light in the presence or absence of acetate, and that glutamine is as effective as ammonium nitrate as a nitrogen source.

The curves in FIGURE 2 compare growth rates in the light in the presence of 0.1 per cent glutamine on medium 2 with and without acetate, and with and without ammonium nitrate. Cells grown on glutamine plus acetate with or without added ammonium nitrate (curves A and B) grow at the same rate as cells on acetate alone (FIGURE 1, curve A), the generation time *g* in both cases being seven hours per division. Cells grown on glutamine in the light without acetate, with or without added ammonium nitrate (curves C and D), grow at approximately the same rate as cells on medium I (FIGURE 1, curve B), 10 hours per division. It is evident from these curves that the over-all growth rates are determined by the presence or absence of acetate, and that glutamine has no apparent effect upon growth except as a nitrogen source.

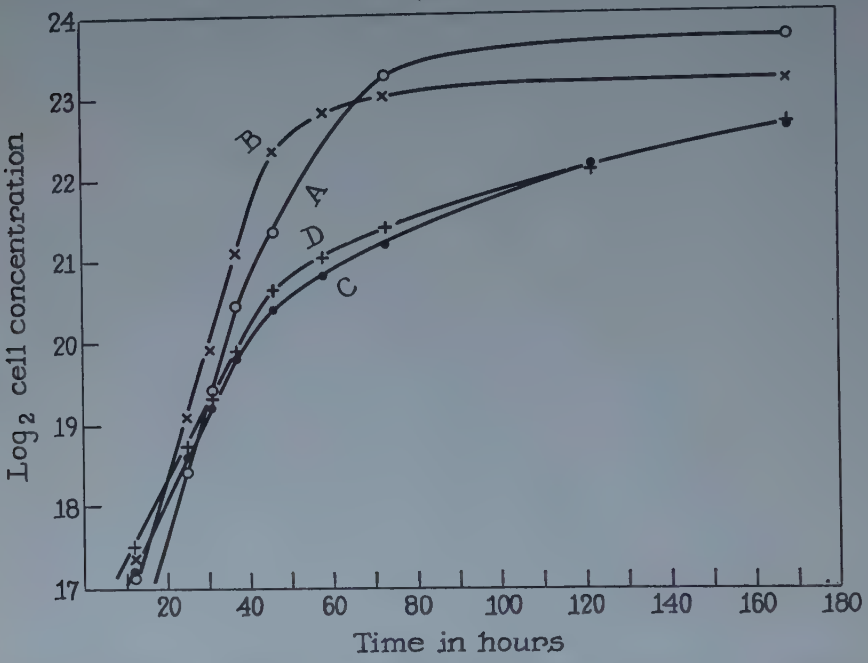

FIGURE 2. Utilization of glutamine in the light as nitrogen source but not as carbon source by *Chlamydomonas reinhardi*. Cells were grown in light in medium II minus acetate minus NH_4NO_3, with the following additions:
Curve A, 0.1 per cent glutamine + 0.2 per cent acetate + 0.03 per cent NH_4NO_3;
Curve B, 0.1 per cent glutamine + 0.2 per cent acetate;
Curve C, 0.1 per cent glutamine + 0.03 per cent NH_4NO_3;
Curve D, 0.1 per cent glutamine.
In Curves A and B, the generation time, *g*, is 7 hours in the log phase of growth. In curves C and D, *g* is 10 hours in the log phase of growth. Inoculum used contained 10^4 cells/ml. grown in medium II in light, in the log phase of growth.

2. *Nutritional Requirements for Zygote Formation*

Nutritional requirements for zygote formation differ from those for vegetative growth. Our studies on the nutritional control of sexuality in *C. reinhardi* will be briefly summarized here. Details will be published elsewhere.[7]

C. reinhardi is a single-celled heterothallic alga with two mating types. It is an isogamous species; *i.e.*, the mating types look alike. A given cell may multiply mitotically in the vegetative state, or may behave as a gamete, sticking to and fusing with a cell of the opposite mating type to form a diploid zygote. No morphological differences between vegetative and gametic cells have been observed, but from the special ability of gametic cells to pair and fuse, it is assumed that biochemical differentiation of a vegetative cell into a gamete must have occurred.

We have found that, with this alga, gametic differentiation can be controlled nutritionally by the level of nitrogen in the medium. Two lines of evidence lead to this conclusion.

(1) Cells grown in the light and taken at the log phase of growth are nongametic. When such cells of the two mating types are mixed, they do not fuse. If the mating types, however, are suspended separately in distilled water in the light, after many hours they gradually become gametic, as shown by the fact that when mixed they do fuse to form zygotes. Cells from older cultures placed in distilled water require progressively less time in the light to become gametic and, finally, the light requirement for gamete formation is lost altogether. Since the light requirement can be met before the mating types are mixed, it is apparent that light is required not for act of copulation, but for some change in the vegetative cells which converts them to gametes (*cf.* Smith[8]).

From this experiment, it was inferred that light might be acting photosynthetically by providing excess carbohydrates to tie up other reserves; in particular, nitrogenous ones, and that nitrogen availability might play a key role. To test this hypothesis, cells were grown in light in a low-nitrogen medium. It was found that they lost their light requirement for gamete formation much earlier in the growth of the culture than did cells grown with high nitrogen. Prompted by this observation, we attempted to cross cultures grown in the dark in a low-nitrogen medium, despite numerous reports that light is required for copulation of *Chlamydomonas*.

We have been able to induce zygote formation in the dark, not only of normal green strains grown in the dark, but also of brown non-photosynthesizing mutants, which must be dark-grown due to accumulation of photosensitizing precursors of chlorophyll which kill cells in the light. Thus, it is clear that a low-nitrogen medium induces differentiation of gametes.

(2) The key role of nitrogen was further shown by studying the reversibility of gamete formation. When active *plus* and *minus* gametes are separately suspended in inorganic nutrient medium, they rapidly dedifferentiate, and will no longer copulate when mixed. When each component of the medium was tested, it was found that only the nitrogen source had this inhibitory effect. Any nitrogen compound which the cells could use for growth was inhibitory to copulation. The nitrogen inhibition could be reversed by washing and resuspending the cells in a no-nitrogen medium in the light, thus, leading again to copulation.

Thus, it appears that (1) nitrogen depletion is essential for gamete formation; (2) light, when required, acts photosynthetically; and (3) in nitrogen-depleted cells, light is not required for any step in the process of zygote formation.*

* Cells are momentarily exposed to dim light in making transfers; the possibility of some effect due to such short exposures to light has not been examined.

Ever since the work of Klebs (1896)[9] expedients, such as staling of the medium, starvation, and suspension in distilled water have been reported to evoke sexuality in a wide variety of micro-organisms. In no instance, has the mechanism been analyzed, but, in several cases studied, the concentrations of both nitrogen and carbon compounds have been implicated. In higher plants, too, there is much evidence that high carbohydrate and low nitrogen favor differentiation of flower buds rather than vegetative buds, but the inaccessibility of the bud primordia has impeded direct analysis of the relationship. In *Chlamydomonas*, we have been fortunate to find an instance in which the controlling roles of nitrogen and carbohydrate, in the induction of gamete differentiation, can be demonstrated so directly. It remains to be determined what is the specific metabolic function of nitrogen in this differentiation, and how general may be the role of nitrogen in controlling the process of sexual differentiation in plants.

Summary

1. The growth rates of *Chlamydomonas reinhardi* have been determined under several nutritional conditions in light and dark, and the maximum rates, about 7 hours per division in the light and about 14 hours per division in the dark, were observed to occur on a simple defined medium with acetate as a carbon source. No other compound was found which served as carbon source for growth in the dark. Glutamine or urea are adequate as sole nitrogen sources for growth in light and dark, but neither can be used as a carbon source.

2. Differentiation of vegetative cells into gametes was found to be controlled nutritionally. Only nitrogen-depleted cells were found to be capable of differentiating into gametes. By depletion of the nitrogen, zygote formation was induced in the dark, in mutant strains which cannot photosynthesize. It is thus possible nutritionally to remove the light requirement for zygote formation in this species. It is postulated that, for gametogenesis, the role of light is photosynthetic. It provides a source of carbohydrate to deplete the available nitrogen.

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ECOLOGICAL IMPLICATIONS OF *IN VITRO* NUTRITIONAL REQUIREMENTS OF ALGAL FLAGELLATES

By Luigi Provasoli and Irma J. Pintner
Haskins Laboratories, New York, N. Y.

Introduction

The productivity of fresh-water bodies and the ocean rests on the algal phototrophs at the base of the food pyramid. To predict the fertility of natural waters it is obviously necessary to learn what governs the proliferation of these algae.

Lucas[20] in 1947, reviewing the evidence supporting his broad concept of "non-predatory" relationships among organisms living in water, emphasized the importance of external metabolites produced by water organisms and pointed out that through these metabolites one kind of organism might affect the growth of others in several ways: (1) by producing necessary nutrients; (2) by removing inhibitory compounds; (3) by excreting inhibitory substances. The present paper describes the growth requirements of several algae in pure culture and, in so doing, documents some aspects of these "non-predatory" relationships.

Certain centric diatoms important in the pelagic phytoplankton were postulated by Harvey[8] to need one or more growth factors. This heightens interest in the observation that the one strain of *Amphora perpusilla*, a neritic pennate diatom, studied by us, requires cobalamin.[12] When coupled with other similar findings detailed in this paper, it brings into sharper relief the problem of the relative incidence of auxotrophy in coastal and oceanic phytoplankton species.

In the course of work with some fresh-water planktonic protists, a remarkable parallelism was observed between *in vitro* results on the effective concentration ranges of nutrients and the composition of natural waters established by chemical analysis, suggesting that the chemical data of the hydrobiologist are a reliable guide to pure culture studies. Conversely, nutritional studies, in revealing that many algae from a variety of environments require vitamins and trace elements, focus attention on these two classes of nutrients which have often been neglected in classical hydrobiological analyses. It would appear, accordingly, that coöperation between hydrobiologist and nutritionist should increase understanding of the factors underlying algal successions and blooms.

Cultural Techniques

General Methods. These generally were the same as those described for *Ochromonas*.[13] Distilled water was passed through a mixed-bed ion exchanger ("Deeminizer"); the purity being controlled by conductivity measurements. Volatile preservative[13] was immediately added to the water, which was stored in polyethylene carboys. The culture vessels were screw-capped tubes with the liners of the caps removed. The cork liners retained objectionable quantities of the detergent used for cleaning, and so induced precipitates in the poorly buffered very dilute media needed for the more delicate fresh-water planktonts.

Technique of Isolation. In attempting to isolate and cultivate an organism belonging to a group never previously grown aseptically *in vitro*, the main problem is to find a medium as complete as possible yet non-inhibitory. Motility is an excellent index of lack of inhibition. Loss of photosynthetic pigments serves as indicator of inhibition for non-motile forms. Before washing or plating (see Pringsheim[24]), preliminary trials of the suitability of media are made by fishing 10 to 20 individuals from the natural waters and putting them into a variety of media and observing their behavior after 6 to 24 hours. For *Synura*, one of the most delicate forms, a peat-mineral medium was developed (TABLE 1) with the motility response as guide (the medium is basically a modified v. Wettstein medium).[38] Other media which have proved useful for isolation of unicellular and colonial Chlorophyceae, for *Ochromonas*, *Cyanophora*, and for a fresh-water *Peridinium* are given in TABLE 2. These media are employed also as conservation media, either as such or slightly agarized (0.2 g per cent).

TABLE 1

ISOLATION MEDIUM FOR *Synura* (MG. PER CENT)

Ca (as NO_3)	0.65	K_2HPO_4	0.4
$MgSO_4 \cdot 7H_2O$	1.0	KCl	0.6
Ca (as Cl)	0.4	Fe (as SO_4)	0.06
$CaSO_4 \cdot 2H_2O$	1.0	Peat extract*	13.0 ml./100 ml.

pH 5.0–5.5; determined with the glass electrode.

The Ca salts were prepared by dissolving $CaCO_3$ in HCl or HNO_3 and driving off excess acid. Since the chloride and nitrate are hygroscopic, the concentrations shown refer to the metal, not the salt, in the final medium.

* Prepared by autoclaving (118–121°C.) black peat with an equal weight of water and filtering, then diluting to the color of "light beer" (see v. Wettstein's directions).[38]

TABLE 2

ORGANIC MEDIA FOR ISOLATION AND CONSERVATION OF FRESH-WATER ALGAE

A. Medium for *Chlorophyceae* and *Cyanophora* (mg. %)		B. Medium for *Peridinium* and *Ochromonas* (mg. %)	
Na_3 citrate$\cdot 2H_2O$	2.0	$Ca(NO_3)_2 \cdot 4H_2O$	10.0
$MgSO_4 \cdot 7H_2O$	2.0	$MgSO_4 \cdot 7H_2O$	2.0
K_2HPO_4	2.0	K_2HPO_4	2.0
Fe (as SO_4)	0.2	Glucose	50.0
Thiopeptone (Wilson)	60.0	Trypticase (B.B.L.)*	50.0
Trypticase (B.B.L.)*	16.0	pH 6.5	
Yeast extract (Difco)	5.0		
pH 6.5			

* Baltimore Biological Laboratory

Rich media favor the growth of bacteria and molds and therefore have not been used for isolation of algae by the plating method. Mineral media have been used almost exclusively from Beijerinch on. Mineral media in effect selected for autotrophs and created the impression that algae, like most land plants, were completely autotrophic.

The designations of nutritional types employed here follow current usage. *Auxotrophs* require growth factors. *Autotrophs* derive all their carbon from CO_2. Van Niel[37] pointed out that there is a continuous quantitative series between complete obligate autotrophs and heterotrophs, and that the reliance on fixed carbon entailed by auxotrophy, while vital to the life of the organism, was only a slight quantitative impairment of autotrophy. To select an extreme example, a photo-autotroph, auxotrophic only in respect to cobalamin, has lost less than a billionth of its autotrophic capacity.

Rich media can be used in the washing technique since accompanying organisms are eliminated mechanically before the wanted organism is inoculated in the rich isolation medium. The advent of the washing technique permitted the discovery of auxotrophic algae. The restriction of the plating technique to mineral media can be partially lifted by adding appropriate antibiotics. As is to be expected, antibiotics proved to be more effective in combination. Mixtures of penicillin and chloramphenicol or streptomycin were useful. *Amphora perpusilla* and other diatoms, for example, were isolated from organic agar media containing penicillin 1,000 units/ml. + chloramphenicol 25 μg./ml. Streptomycin, however, is especially poisonous to blue-green algae[5, 22, 27] (1 unit or less may be poisonous) and to a lesser degree to Chlorophyta (poisonous range 1–50 units/ml.). Certain *Euglenas* are not inhibited by high concentrations of streptomycin but may become permanently colorless.[26]

Determination of Growth Requirements. Once the organism is obtained in pure culture, much information can be gained in one step by inoculation of a variety of media ranging from mineral to increasingly complex, and at different dilutions. If complex media are needed (peptones, soil extract, yeast autolysate, *etc.*) the next step is to identify the active constituents. This is done by stepwise substitution of natural materials with a mixture of better-defined preparations (acid-hydrolyzed gelatin and casein; alkali-hydrolyzed yeast nucleic acid, *etc.*) and pure compounds in the form of a mixture of known vitamins and likely carbon sources. This screening procedure is illustrated in other papers from this laboratory.[3, 34]

One of the frequent difficulties encountered in applying screening procedures to planktonic organisms is that they tolerate very low overall concentrations of media. The ever-present problem is to supply materials in adequate yet non-toxic concentrations. Special care has to be exercised not to overlook mineral deficiencies induced in the process of replacing crude materials with pure compounds. To avoid precipitates of calcium, iron, and phosphates, it is helpful to add a solubilizing metal-complexer. Experiments are easier to interpret if the complex-former is unmetabolizable and does not penetrate the organism. In using such metal-buffering agents, essential trace elements ordinarily adequately supplied as impurities in the "chemically pure" minerals, become inadequate. The need then becomes strikingly evident, and the proper concentrations of these trace elements have to be determined.[28] Occasionally, growth factors can be required which may be new to biochemistry.

Chemically-Defined Media. Media developed for determination of the growth factor requirements of *Peridinium* sp., *Gyrodinium* sp. *Cyanophora paradoxa*, and *Synura* sp. are given in TABLE 3. The *Gyrodinium* is marine and therefore its medium is a substitute for enriched seawater. Its isolation medium consisted of seawater enriched with NO_3, PO_4, Fe, and soil extract.

The identification of requirements for growth factors and trace elements, and the recognition of optimal ratios and concentration boundaries for major inorganic and organic nutrients are treated here as separate topics for the sake of arriving at a general picture of nutritional specializations in algae. Obviously they are some of the interrelated factors to be considered in devising good artificial media. The identification of the nutritional requirements in all these categories for *Peridinium* and *Cyanophora* is advanced enough to allow reproducible macroscopic growth in a month. The other organisms are, as yet, unsatisfactorily known in one or more categories. It has not been determined whether the medium for *Synura* will support indefinitely subculturable growth.

TABLE 3

CHEMICALLY DEFINED MEDIA FOR IDENTIFICATION OF VITAMINS (MG. PER CENT)

	Cyanophora paradoxa	*Peridinium* sp.	*Synura* sp.	*Gyrodinium* sp.
Ethylenediamine tetraacetic acid (EDTA)		30.0	5.0	20.0
Citric acid·H_2O	30.0			
$NaNO_3$			2.0	
NH_4NO_3	3.0			
KNO_3		5.0		10.0
KH_2PO_4	10.0	2.0	1.4	2.0
$MgSO_4 \cdot 7H_2O$	5.0	10.0	2.0	30.0
*$MgCl_2 \cdot 6H_2O$				300.0
NaCl				2400.0
$CaCO_3$	10.0	10.0		
Ca (as Cl)		4.0	1.3	5.0
KCl			1.0	30.0
Fe (as NO_3 + Cl)	0.3	0.2	0.07	0.3
Zn (as Cl)	0.01	0.08	1.0	0.4
Mn (as Cl)	0.015	0.08	0.2	1.0
Mo (as Na salt)	0.01	0.05	0.001	0.05
Co (as Cl)	0.0004	0.001	0.003	0.003
Cu (as Cl)	0.0001	0.0001	0.0005	0.0003
†"S1 metals"				0.5 ml./100 ml.
Boron (as H_3BO_3)				0.2
Na H glutamate	15.0	100.0	10.0	2.0
Glycine		70.0		
DL-Alanine		70.0		
Na acetate·$3H_2O$	10.0		4.0	
Glucose		50.0		
DL-Lysine HCl				1.0
DL-Leucine				0.2
	pH 6.3	pH 6.2	pH 5.5	pH 7.5

* Concentration in stock solution standardized by chloride determination.

† *"S1 metals":* 1.0 ml. of the mixture contains the following metals: Sr (as Cl) 1.3 mg.; Al (as Cl) 0.05 mg.; Rb (as Cl) 0.02 mg.; Li (as Cl) 0.01 mg.; I (as KI) 0.005 mg.; also Br (as NaBr) 6.5 mg.

Vitamin concentrations needed for growth: Cobalamin is added for *Gyrodinium* and *Cyanophora* at the 0.1 μg.% level; for *Peridinium*, 0.06 μg. per cent; and for *Synura*, 0.15 μg.per cent. The need for other vitamins is under study.

Sources of metals. The Zn was prepared by dissolving Johnson-Matthey purified zinc rod in distilled HCl. The Co and Mn were Johnson-Matthey "spec-pure" solutions of the chlorides. The Fe solution was prepared by dissolving "spec-pure" metal in aqua regia compounded of distilled HCl and HNO_3; excess acid was then evaporated off.

Growth-Factor Requirements

The findings to date for the flagellated and non-flagellated algae are summarized in TABLE 4. Many chlorophyceans, not included in this table, are maintained in algal collections on mineral agar. The great majority of these are probably non-auxotrophic, but a more rigorous avoidance of chemical contaminations (thiamine, cobalamin, *etc.*) from agar, cotton, and distilled water[31] might reveal auxotrophy in a few. Several *Euglenas* are omitted, since it is not clear whether or not a sufficiently rigorous technique was applied. It is not unlikely that all euglenids require cobalamin and thiamine.

Four general conclusions may be drawn from TABLE 4:

TABLE 4
INCIDENCE OF GROWTH-FACTOR REQUIREMENTS IN ALGAE
(Numbers in parentheses are references)

Species	Vitamins needed	Thiamine	Cobalamin	Other
CHLOROPHYTA				
Chlamydomonas agloëformis	0 (7)*			
Chlamydomonas chlamydogama	+ (23)		+	Histidine
Chlamydomonas moewusii	0 (11)			
Chlamydomonas sp. ("marine")	0 (23)			
Chlorogonium elongatum	0 (7)			
Chlorogonium euchlorum	0 (7)			
Coelastrum (*morus* ?)	+ (15)	+		
Haematococcus pluvialis	0 (7)			
Lobomonas pyriformis	0 (7)			
Lobomonas rostrata	+ (15)		+	
Polytoma caudatum	+ (7, 18)	+		
Polytoma obtusum	0 (7, 18)			
Polytoma ocellatum	+ (7, 18)	+		
Polytoma uvella	0 (7, 18)			
Polytomella caeca	+ (7, 18)	+		
Prototheca zopfii	+ (1)	+		
Selenastrum minutum (E. A. George strains)	0 (15)			
Selanastrum (*minutum* ?)	+ (15)	+		
CHRYSOPHYTA				
Amphora perpusilla	+ (12)		+	Uracil ?
Nitzschia closterium f. *minutissima*	0 (10)			
Nitzschia putrida	0 (12)			
Ochromonas malhamensis (3 strains)	+ (13)	+	+	Biotin + histidine
Poteriochromonas stipitata	+ (13)	+	+	Biotin + histidine
Synura sp.	+	?	+	?
Syracosphaera carterae	+	?	?	?
EUGLENOPHYTA				
Euglena gracilis vars. *typica*, *bacillaris*, *urophora*	+ (14)	+	+	
Astasia longa (= *klebsii* Von Dach)	+ (36)	+	+	
E. gracilis, streptomycin-bleached	+ (31)	+	+	
E. pisciformis	+ (7, 18)	+	?	
E. viridis, *E. stellata*	+ (11)	+	+	
PYRROPHYTA				
Chilomonas paramoecium	+ (7, 18)	+		
Cryptomonas ovata var. *palustris*	+	?	+	?
Cyanophora paradoxa	+		+	
Gymnodinium splendens	+ (35)†		+	
Gyrodinium sp. (marine)	+	?	+	?
Peridinium sp.	+	+	+	?

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* Most of the earlier literature on auxotrophy in algal flagellates is reviewed in reference (7). Where the reference is not given, the data refer to the present paper.

† Personal communication from Doctor Beatrice M. Sweeney

(1) Algae, like autotrophic bacteria, may be divided into auxotrophs and non-auxotrophs.

(2) Thiamine and cobalamin (vitamin B_{12}) are needed by the majority of the auxotrophs. The auxotrophic forms occur in all groups of algal flagellates and auxotrophy does not correlate with any particular environment: Most species of *Polytoma*, *Astasia*, and *Euglena* are found in environments rich in organic matter (polysaprobic); *Synura* and *Cryptomonas* live in waters poor in organic matter (oligosaprobic); *Gyrodinium*, *Nitzschia* (2 species, both non-auxotrophic, one, the colorless *N. putrida*, completely heterotrophic), and *Amphora* are marine. Considering the multiplicity of metabolites in nature, this pattern of dependency on cobalamin and thiamine is remarkably stereotyped and leads one to wonder whether this expresses a deep-seated evolutionary tendency in their physiology.

(3) Other vitamins may be needed besides the seemingly predominant thiamine and cobalamin; *e.g.*, biotin is required by *Ochromonas malhamensis* and *Poteriochromonas stipitata*. It might be expected that additional vitamins will be found to be required as the number of species of algae in pure culture increases.

(4) Auxotrophy is present in organisms having different degrees of heterotrophy. It may be the only heterotrophic requirement, as it appears to be in *Synura*, or it might be only one element of a many-sided heterotrophy: *e.g.*; *Euglena gracilis* utilizes various substrates, and *Ochromonas* and *Poteriochromonas* are photosynthetic phagotrophs exemplifying the coexistence of plant and animal nutrition, phagotrophy being the principal mode of heterotrophy in animals.

Auxotrophy in algae was first detected by Lwoff and Dusi[19] in several genera of colorless chlamydomonads, in a colorless cryptomonad, and in *Euglena pisciformis*. References to the earlier literature are given by Lwoff[18] and Hall.[7]

The question arises as to the extent to which auxotrophy is a sign of a heterotrophic tendency in algae. A better understanding may be obtained by reviewing the evolutionary trends in algae leading to the development of the animal forms, *i.e.*, the Protozoa. The new data on the physiology of *Euglena* and *Ochromonas* permit elaboration of the physiological implications that Lwoff[17] attached to the morphological evolutionary tendencies recognized and systematized by Pascher and by Fritsch.[6] These are outlined in TABLE 5. It will be noted that each algal group exhibits certain conspicuous morphological tendencies. These, in so far as they affect the photosynthetic apparatus, are bound to be reflected in a changed nutritional pattern. Obviously, when photosynthesis is lost, survival demands a compensatory heterotrophic ability. In different algal groups heterotrophy may assume either of two forms: In phytomonads, osmotrophy; in euglenids and all other groups, osmotrophy and phagotrophy. The clearest index of an adequate heterotrophy is the ability to grow in darkness, as is shown by *Chlamydomonas agloëformis*, *Chlorogonium euchlorum*, *Euglena gracilis*, *Ochromonas malhamensis*, *Poteriochromonas stipitata*, *etc.* Many algae, however, utilize fixed carbon without being able to grow in darkness. Their heterotrophy is less efficient than is that of the dark-growing species. The existence of facultatively heterotrophic, photosynthetic organisms clearly indicates that facultative heterotrophy precedes loss of photosynthetic power and can be considered as a sign of an evolutionary tendency toward animal nutrition. In nature, organisms with dual capacities—phototrophy and heterotrophy—are abundant. Depending on external conditions, one or the other mode of nutrition may predominate. It does not necessarily follow that pigmented organisms with an efficient heterotrophy have a weakened photosynthesis. Cramer and Myers[4] have shown that *Euglena gracilis* var. *bacillaris*, when provided with sufficient light and CO_2, photosynthesizes at least as vigorously as *Chlorella*. Furthermore, autotrophic growth under these conditions equals the best heterotrophic growth. The relative importance of autotrophy, osmotrophy, and phagotrophy in *Ochromonas* and *Poteriochromonas* remains to be determined. In view of the tendency already noted of all algal flagellates to lose photosynthetic pigments, the widespread occurrence of heterotrophy and auxotrophy in pigmented forms is not surprising. The minute heterotrophy represented by auxotrophy in algae is nevertheless all-important in determining which algae will multiply under a given set of circumstances. It is also tempting to look upon auxotrophy as one of the forerunners of a more pronounced heterotrophy.

TABLE 5

GROUP TENDENCIES IN ALGAL FLAGELLATES*

	Tendency to loss of:		Tendency to phagotrophy
	chlorophyll	plastids	
Colonial Volvocales	none	none	none
Chlamydomonadaceae	pronounced	none	very rare
Polyblepharidaceae	pronounced	none	none
Euglenoids	pronounced	none	doubtful
Peranemids	total	widespread	total
Cryptomonads	rare	rare	very rare
Chrysomonads	widespread	widespread	widespread
Dinoflagellates	pronounced	pronounced	widespread

* Revised from Lwoff,[17] p. 216.

Presence of Growth Factors in Natural Waters. It is a reasonable assumption that if an organism requires a growth factor *in vitro*, then this metabolite or its physiological equivalent should be found in significant amount in the environment. Only the actual determination of the seasonal variation of these vitamins in nature will enable detection of the times when these vitamins actually become the limiting factors for growth. A promising start has been made. Robbins *et al.*[29] charted the fluctuation of cobalamin content of a small pond which had shown *Euglena* blooms. The amounts of cobalamin found were high enough to satisfy the cobalamin requirement of *Euglena*. Hutchinson[9] earlier showed that a thiamine cycle existed in a pond but did not attempt to correlate this with any particular organism.

The question arises as to the identity of the principal vitamin-producers in nature. Robbins *et al.*[29] found that soil extract contains appreciable amounts of cobalamin and that 40 per cent of the bacteria from mud isolated at random excreted cobalamin. Since cycles of metabolites in soil parallel those in water, Lochhead and Thexton's[16] demonstration is apropos that 70–84 per cent of the bacteria isolated at random from soil are cobalamin producers and that 14 per cent require soil extract. It is interesting to note that half the bacteria initially requiring soil extract were satisfied by cobalamin.

The data of TABLE 4 provide examples of the importance of external metabolites—of "non-predatory" relationships—existing in the water environment. Only a small part of the cobalamin cycle is known. The literature indicates that many other organisms participate in it. As with bacteria, algae include cobalamin-dependent organisms and cobalamin producers. Robbins *et al.* found that an extract of 7 fresh-water blue-green algae grown in mineral media contained relatively high amounts of cobalamin. The uninoculated medium showed no activity. Extracts of several red and brown algae contained cobalamin, especially *Ceramium rubrum.* Since the algae were obtained directly from the shore there is some doubt as to whether all the cobalamin was theirs. Oysters and clams were found by Robbins *et al.*[30] to contain large amounts of cobalamin, suggesting that algal flagellates, an important item in their diet, are either producers or accumulators of cobalamin.

Requirements for Trace Metals

Requirements of Synura. This organism, often abundant in unpolluted waters such as reservoirs, has long challenged the nutritionist as well as the hydrobiologist. It is selected here to exemplify the problems faced in culturing a planktont requiring very dilute media. It was cultured by Mainx[21] in a highly diluted soil extract. Rodhe[32] grew it in a very dilute mineral medium (his "medium VIII"; see TABLE 6) supplemented with an extract of soil or lake sediment. Ashed extracts were ineffective. Our medium (TABLE 3) was developed by replacing peat extract with a mixture of known vitamins. Since the medium is prone to precipitate, EDTA, an efficient solubilizing chelating agent, was added. This procedure revealed, as expected, deficiencies in trace metals which were satisfied by finding the suitable concentration of a series of different trace metal mixtures. Then, keeping constant the levels of trace elements, the major elements (Ca, Mg, K, NO_3, PO_4, and Fe) were adjusted to levels permitting better growth. Using, in turn, this information, the suitable concentration of each of the trace metals, previously added as a mixture, was determined. Inoculation of the experiments, at first, was from stock cultures maintained in the isolation medium, later from preceding experiments. Growth in defined media was better than in the original peat medium but decreased after repeated transfers. This indicated that the defined medium is still incomplete and that carry-over of factors from peat may play a role. In an attempt to identify other substances needed for growth, experiments are under way to identify the effective vitamins in the dilute vitamin mixture used. Cobalamin is required; but additional vitamins may be needed. The effect of exogenous carbon sources was also explored. In unagitated cultures, not supplied with extra CO_2, growth was unaffected by single additions of glutamate, acetate, fumarate, glucose, glycine, and alanine. It should be noted that Zn is required at a very high concentration as compared with Ca, Mg, and K, which are usually designated as major nutrients. *Euglena gracilis* var. *bacillaris*, supplied with a 10-fold higher concentration of EDTA, does not require for heavy growth more than 2.0 mg. per cent of Zn at pH 5.0.

Some acute problems are presented by dilute media; *e.g.*, adequate pH buffering is difficult to achieve because of the low tolerance to phosphate and osmotic pressure. Aconitic, trimesic, and succinic acids show promise as buffers in the acid region. Dilute media are especially difficult to reproduce, since environmental chemical contaminations from glassware, distilled water, and especially from "c. p." chemicals, may be of the same magnitude as the nutrients added intentionally. Media built around EDTA are an advance toward reproducibility. EDTA's metal-sequestering abilities make it, at the same time, a solubilizing agent preventing precipitation and a metal-buffering agent raising the threshold of metal availability and toxicity. Hit-or-miss dependence on "chemically pure" salts as a source of essential trace elements is thus minimized; now the trace elements have to be added as such.

Comparison of artificial media and lake waters. Chu and Rodhe were the pioneers in devising artificial dilute media for many planktonts. Chu[2] developed 16 different media of which "No. 10" permitted growth of several diatoms, a blue-green alga, a desmid, and *Botryococcus*. His other media were developed for the various single species. Rodhe's "No. VIII" served for cultivation of several Chlorococcales, Volvocales, Heterokontae, and desmids. Chu's and Rodhe's media were developed through determination of the concentration range for each constituent. The species cultivated by them are almost certainly complete autotrophs since growth factors were not supplied. As mentioned earlier in the discussion of growth factors, auxotrophy cannot be excluded until a rigorous technique is employed. However, Rodhe's failure to grow *Synura*, which requires cobalamin, indicates that his mineral media were not significantly contaminated with cobalamin, one of the most powerful and ubiquitous of growth factors. Our media, developed independently, ended by being as dilute as theirs. Our experiments with *Cryptomonas ovata* var. *palustris* indicate that, as a representative planktont, it has a very low osmotic tolerance. As little as 30 mg. per cent NaCl was inhibitory. These results also agreed with tolerances to neutral salts of organic acids.

TABLE 6 shows the striking similarity of the three mineral media and the similarity existing between them and Rodhe's standard mineral composition of lake waters having a similar content in total solids. This parallelism between laboratory results and analyses of natural waters validates the idea that laboratory findings in respect to growth factors and mineral requirements are directly relevant to ecological problems. As already mentioned, use of a chelator reveals sharply the indispensability of trace elements. The finding of Chu that addition of trace elements was unnecessary implies that the impurities present in his major chemicals were adequate. Rodhe made an extensive study of the precipitation—*i.e.*, unavailability—of Fe and found that addition of a Fe citrate-citric acid combination prevented precipitation. Although citric acid is only a moderately strong metal-binder and was used in very small amount, the need for Mn became evident. The use of chelators in the laboratory parallels the situation in natural waters where humic acids are among the most important, almost unmetabolizable, metal-buffering agents. Since planktonic organisms such as *Synura*, *Peridinium*, *Cryptomonas*, and *Trachelomonas* have been shown to require trace metals, more importance should be assigned to trace metals as ecological factors. A recent paper by Rodhe[33] signalizes the increasing interest in the trace element content of natural waters.

Phosphate requirements. An *in vitro* result that cannot yet be correlated with ecological data is the requirement for phosphorus. In fact, the concentrations needed in synthetic media far exceed those normally present in nature. For instance, Chu and Rodhe[32] found that *Asterionella formosa* is indifferent to 10–20 μg. *P*/liter. Maximal growth was obtained at about 1,000 μg. P/liter. Rodhe found that, in Lake Erken, where this organism is common, the P content scarcely attains 10 μg./liter. When his artificial inorganic basal medium was replaced by lake water (sterilized by filtration), maximum growth was obtained with the extremely small addition of 4–10 μg. P/liter. This striking discrepancy in utilization of P appears to support Rodhe's assumption that lake waters contain one or more factors facilitating the use by *Asterionella* of P at very low concentrations. A peculiarity in P utilization by *Cryptomonas ovata* may be relevant. In one experiment, different PO_4 optima were observed on varying the concentration of magnesium sulfate. For $MgSO_4 \cdot 7H_2O$ at 2.0 mg. per cent, the optimal level of KH_2PO_4 was 1.0 mg. per cent; while for $MgSO_4 \cdot 7H_2O$ 0.5 mg. per cent, the optimal level of KH_2PO_4 was 0.4 mg. per cent. If these preliminary results prove repeatable, it will be determined whether this varying response to KH_2PO_4 depends on the ratio of Mg to K. In any event, Rodhe's experiment should stimulate a search for factors

TABLE 6

COMPARISON OF LAKE WATER OF STANDARD COMPOSITION AND ARTIFICIAL MEDIA
(Mg. per liter)

Elements	Rodhe's Standard Composition*	Chu No. 10	Rodhe No. VIII	Synura Medium†
N	0.5 –0.6‡	7.0	10.2	3.3
P	0.03–0.05‡	1.8–9.0	0.89	3.1
Na	4.6	19.0	7.5	5.4
K	1.7	2.2–4.4	2.2	9.0
Mg	2.7	2.5	1.0	2.0
Ca	16.3	10.0	14.7	13.0
Fe	0.4–1.2‡	0.27	0.18	0.7
Mn	0.02–0.14‡		0.01	†
Total Solids	98.0	115–120	181	102–179**
Conductivity ($\psi.10^{-6}$)	120.0			

* Values from TABLE 2 of Rodhe, W., 1949. The ionic composition of lake waters. Proc. Intern. Assoc. Limnol. **10:** 377–386.

† See TABLE 3 for concentrations of EDTA and minor elements; 0.2 ml/100 of a vitamin supplement was added (formula in Cowperthwaite *et al.*[3]).

‡ Variation intervals, calculated from Rodhe's[33] tables, for a conductivity of 120.

** In the higher value the sodium salts of glutamic and acetic acids are not included (*Synura* can grow without this addition).

In the lower value glutamate, acetate, trace metals and EDTA are not included.

in natural waters permitting growth at low P concentrations. P is undoubtedly one of the important natural limiting factors for most algae. Rodhe found, on the other hand, that blooms of *Dinobryon* and *Uroglena* appeared only when the P level was below 5 μg./liter and, experimentally, their growth was inhibited upon addition of 5–10 μg. P/liter. Our results with *Peridinium*, *Gyrodinium*, *Synura*, *Cryptomonas*, and *Trachelomonas* further document the well-established fact that the need for PO_4 varies among algae, and indicate, moreover, that some plankton organisms have a surprisingly narrow optimal PO_4 range. This last point may be important in ecology.

Discussion

The water environment is the one in which metabolites are interchanged most efficiently. It is to be expected that the interdependent growth of the different groups of water organisms should sensitively reflect the excretion and consumption of metabolites. Undaunted by new intricacies, we should envisage all the possibilities in these relationships, and not hesitate to follow Lucas's lead in constructing theoretical frameworks upon which to hang data. In the present paper, only a few aspects of the nutrition of photosynthetic forms are considered. It is possible, nevertheless, to state more definitely some of the interdependencies based upon "external metabolites": (1) the interchange of growth factors; (2) the lowering of inhibitory concentrations of several major mineral nutrients, especially PO_4; and (3) the preferential utilization of minerals, including trace metals, may condition waters, bringing their concentrations into the optimal zones for succeeding forms. The practical aim—to predict algal successions and blooms—may be achieved through a comprehensive knowledge of vitamin cycles as well as mineral cycles. An immediate problem is to trace the thiamine and cobalamin cycles. In fresh-waters, these can be worked out by applying techniques such as those used by Hutchinson and by Robbins *et al.*, but tracing these vitamins in the ocean requires the exploitation of new assay organisms and concentration techniques.

The role of other organic materials as ecological factors remains problematic at the moment. It is likely that, in waters high in organic content, such as sewage, organic compounds may serve as substrates for photosynthetic organisms endowed with heterotrophic abilities. A familiar example of this utilization of substrates is presented by the algae which form blooms in sewage lagoons and similar polysaprobic environments. The algae, *e.g.*, *Euglena gracilis* and *Chlorella*, isolated from these habitats are well endowed with heterotrophic abilities.

The coastal waters of well-vegetated, well-populated land masses should be comparatively rich in organic matter. Many phytoplanktonts appear to be coastal rather than pelagic—an indication that the land may be a significant source of essential metabolites.

Summary

Chemically defined media were devised for *Peridinium* sp., *Cyanophora paradoxa*, *Synura* sp., and a marine *Gyrodinium*. All require cobalamin (vitamin B_{12}). Possible additional growth-factor requirements are under investiga-

tion. The occurrence of auxotrophy in algae is reviewed. All algal groups and environments contain auxotrophic forms. Auxotrophy is more prevalent in algae than previously recognized. Planktonic algae often require media very low in solutes. These media correspond in composition to natural waters. The need for several trace elements, notably Zn, Mn, Cu, and Co became evident upon use of a metal-complexer (EDTA) as a solubilizing metal-buffer.

The need *in vitro* of growth factors and trace elements, and the narrow optimal ranges of phosphorus, suggest that all these are significant ecological factors. From the mapping of the cycles of growth factors and trace elements, there would accrue a better understanding of the occurrence of blooms and the succession of species in nature.

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NUTRITION OF SOME PHAGOTROPHIC FRESH-WATER CHRYSOMONADS*

By S. H. Hutner, L. Provasoli, and Joyce Filfus†
Haskins Laboratories, New York, N. Y.

The chrysomonads are a vast array of brown-pigmented fresh-water, soil, and marine flagellates. Colorless phagotrophic counterparts of several of the pigmented species are common in nature, *e.g.*, *Monas* and *Oikomonas.* The minimal nutritional requirements of 4 fresh-water strains belonging to 2 species are described here. No chrysomonad previously has been grown in chemically defined media. These strains ingest particulate food and are therefore animals (protozoa) and, being to some extent phototrophic, they are plants (algae) as well. They can live indefinitely in darkness or in light on high-molecular dissolved food, as well as in low-molecular chemically defined media. This extraordinary versatility, first thoroughly documented in the pioneering pure culture study of Pringsheim,[9] is accompanied by a remarkable tolerance of variations in media and exceedingly dense growth in appropriate media, including certain simple "synthetic" media [one cannot unambiguously call "synthetic" a medium containing cobalamin (vitamin B_{12}), whose complete structure is still to be ascertained]. This ease of cultivation—in which respect these chrysomonads contrast sharply with some of the planktonic chrysomonads[10]—suggests that the biochemical basis of an intermeshed phagotrophy and phototrophy can profitably be investigated in them.

Pringsheim[9] reviewed the scanty previous work. He demonstrated an active phagotrophy in the same strains on which the present paper is based: They ingested starch grains, oil droplets, casein particles, bacteria, and small algae—including each other. Glucose was utilized in the presence of complex natural materials such as peptone. Liver extracts were strikingly stimulatory. Dark growth was improved by aeration and paralleled that in light. Dark-grown individuals were pale. Cultures accumulated a water-soluble sepia or almost black substance which was also produced by pale growths in the dark.

With chemically defined media—the subject of this paper—it should be possible to explore systematically their ability to utilize high-molecular sources of energy and carbon, nitrogen and amino acids, biotin, thiamine, and cobalamin.

To identify the growth requirements of aerobic phototrophs, culture methods are needed which combine adequate illumination with freedom from significant chemical contaminations. Such considerations apply with special force to these chrysomonads. Growing densely, they need much illumination. Hence they were cultured in shallow layers of medium, and their sensitive response to certain widely-distributed growth factors such as biotin and cobalamin spurred the development of methods to minimize the confusion introduced by the vitamin-excreting microorganisms flourishing in stored distilled water, in solutions of C. P. reagents, and in obviously putrescible materials, such as extracts of yeast and liver extracts (*cf.* Robbins *et al.*).[11] It seems worth while to describe these procedures. Many of the details described in previous publications have been modified or require amplification.

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† Present address: Division of Bacteriology, University of California, Davis, California.

Glassware. Borosilicate glassware (Corning and Kimble) was used. After trial of various commercial synthetic detergents, a cleaning mixture (A. H. Thomas Co.) containing Na_4 ethylenediamine tetraacetate ["Versene"; designation in *Chemical Abstracts*: (ethylenedinitrilo) tetraacetate], was adopted, inasmuch as calcium, iron, and manganese deposits were hardest to remove. Chromic-sulfuric cleaning mixtures were inferior, especially for removing greasy deposits, and needed exhaustive rinsing to minimize the adsorption on glass of the toxic chromate ion.[12] Aluminum pots were used for cleaning. The detergent was brought to a boil; the glassware thoroughly rinsed in tap water, and inverted to drain dry. New York City tap water seems to have negligible amounts of the growth factors of interest in the present investigation (thiamine, biotin, and cobalamin). Even if present originally, they would be destroyed by the chlorine added to the water since they are inactivated by oxidation and halogenation. No discrepancies were detected with those experiments in which glassware was rinsed with distilled water. Pipettes were long a serious problem; for they were prone to retain air bubbles and be incompletely rinsed, and their fragility made desirable the least possible handling. A satisfactory procedure was devised: Immediately after use the pipettes were plunged tip down into detergent, then collected in a stainless steel basket (Technicon) and the basket plunged into detergent contained in a stainless steel cylinder (Metalsmiths, Orange, N. J.), upright on a hot plate. The detergent solution was brought to the boil, thus subjecting the vertically-held pipettes to violent ascending convection currents. The carrier basket was then rinsed in an automatic device (Technicon) and the pipettes then allowed to drain dry. Pipettes for aseptic operations were autoclaved in individual Pyrex tubes open at both ends. One end of the outer tube was crimped to retain a permanent plug of Pyrex glass wool. The other end had a cotton plug. After autoclaving, the damp pipettes and outer tubes dried on standing overnight at room temperature after being placed near the lamps used for illuminating cultures. The cotton plugs were used over and over. This procedure eliminated the tedious wrapping of individual pipettes in paper and obviated the greater risk of contamination when pipettes are sterilized together in the same container. Sterilization by dry heat contaminates pipettes with decomposition products of cotton. Cotton soon disintegrates under this treatment.

Sterilization. All media were autoclaved for 25–35 minutes at 118–122°C. This severe treatment insures killing the unusually resistant spore-forming bacteria occasionally present in natural materials, especially in agar. Also, it volatilizes away the preservative added to all nutrient solutions except those to be sterilized by filtration. This preservative has the following composition by volume: chlorobenzene, 1 part; 1,2-dichloroethane (ethylene dichloride), 1 part; 1-chlorobutane, 2 parts. Despite the high boiling point of chlorobenzene (132°), it is driven off completely upon autoclaving. About 1 per cent preservative was added to aqueous solutions; preservative being replaced as needed. Stock solutions later showing neither droplets nor odor of preservative were discarded as possibly contaminated. Occasionally, the preservative formed emulsions difficult to distinguish from the cloudiness of microbial growth. Gentle warming breaks these emulsions. This preservative has been used for three years without failure. Less drastic autoclaving would, of course, require a lower-boiling preservative.

Culture Technique. Experimental media in light were distributed in 10 ml. portions in 25-ml. Erlenmeyer flasks capped with flat-bottomed 24 x 24 mm. glass thimbles. Beakers do not fit properly. Dark cultures require more exogenous oxygen and, therefore, the same volume of medium was distributed in 50-ml. flasks capped with loosely fitting aluminum cups (A. S. Aloe Co.). The flasks were arranged in Pyrex kitchenware trays. After autoclaving, the trays were allowed to cool for 5 to 6 hours in the closed autoclave. The trays were removed and the flasks inoculated. A second tray was then inverted over the tray bearing the flasks and the joint between the trays was sealed with transparent cellulose tape.

Light. In most experiments, 1400–5500 lux was provided by an overhanging bank of four 40-watt "warm white" (3000°K.) fluorescent lamps. The trays were stacked under these lights and rearranged from time to time for even illumination. This illumination was ample for all but very dense cultures (those with optical densities >3.0 or so, measured with a red-sensitive phototube). Pringsheim noted that these chrysomonads do not require much light. Elimination of cotton plugs has advantages. Overhead lights could be used without undue shading of cultures, and the tedious manipulation of plugs was eliminated along with the need to launder plugging cotton to remove growth factors. Illumination from below or the sides exposes cultures to more heat than does illumination from above, unless the lamps are shielded. Dispensing with plugs required that flasks be cooled completely in the autoclave before inoculation. The wet surfaces of the single-wall, autoclave, trays, and culture vessels, acted as dust traps, assisted by the slow cooling and the very gradual bleeding in of air. The elapsed time for an experiment is increased by this slow cooling, but labor is lessened. Few contaminations were encountered with this method.

Most cultures were grown at 27–31° C., the optimum range. Growth is sharply slowed above 33°. In hot weather, experiments were conducted in a refrigerated room at 24–26.5°.

Sterility tests. Conventional bacteriological sterility-test media were not depended upon for detection of macroscopically inapparent contaminants. Many media contained particulate matter difficult to distinguish from bacteria, yeasts, or other microorganisms. Furthermore, as cultures aged, the disintegration of the chrysomonads produced debris easily masking contaminants. Consequently, sterility tests were incorporated into the ground plan of the experiments. Nearly all experiments included media containing complex natural materials at several concentrations. A diversity of materials was used in the course of the investigation. This procedure was not deemed sufficient. One class of potential contaminants might, for example, require materials destroyed on autoclaving and excreted by living chrysomonads and other organisms. Another class of contaminants might grow well only in very dilute media and not at all in natural media containing the usual concentrations of peptone, meat infusions, and the like. Many water bacteria are in this category. In consequence, wet mounts were examined microscopically, while inocula were examined with special care. At times, the chrysomonads were inoculated into dilute semi-solid media including some suitable for growing many planktonic organisms, since many microorganisms grow poorly on the surface of stiff agar, and others grow poorly wholly submerged. What was perhaps the most sensitive test for contaminants was developed as a by-product of the identification of the growth factors. Because of steady cropping, a contaminant in a phagotroph culture may remain inconspicuous. The early literature on ciliates records one such failure to recognize a bacterial contaminant. If the phagotroph has a need for one or more growth factors which can be measured quantitatively, then any increase in the blank not accounted for as carry-over from the inoculum, as determined by serial subculture, would arouse the suspicion that the growth factors were being obtained at least in part from foreign food organisms synthesizing these factors. In effect, therefore, the determination of the quantitative response to biotin, thiamine, and cobalamine, both as pure compounds and in natural materials, performed from time to time as part of studies of the specificity of these requirements, constituted sterility tests.

Culture media. Distilled water from a block tin apparatus was used without further treatment and was consumed within a week. Polyethylene bottles have recently been used for storage. Unlike glass, they do not contribute minerals to the water and therefore keep down the growth of unwanted organisms.

On the basis of several extensive experiments, a conservation medium was devised which supports prolonged dense growth of these chrysomonads, yet is so dilute as to serve as a source of inocula bearing a minimum of carry-over of nutrients, and thus largely obviates the need for washing inocula. In order to select against osmotrophs (organisms living on dissolved nutrients) over phagotrophs, attention was concentrated on media composed preponderantly of high-molecular and particulate materials. These included, in powdered form, whole milk, skim milk, whole liver, and alcohol-extracted liver. The following medium was finally adopted:

powdered skim milk	0.2%
trypticase (Baltimore Biological Laboratory)	0.05%
sucrose	0.1%
pH 6.4–5.9	

This medium is a compromise between the, as yet, conflicting desiderata of obligate digestion and phagotrophy on one side, and rapidity of growth on the other. The above-mentioned undigested materials used by themselves support heavy growth only at high levels and multiplication is slow. The low-molecular carbohydrate in the medium is perhaps objectionable from the standpoint of maintenance of phagotrophy. Studies of the utilization of high-molecular polysaccharides akin to the constituents of the cell walls of bacteria and algae may suggest a better carbohydrate. Starch, as noted by Pringsheim, is vigorously hydrolyzed. Because of the cobalamin requirement, only particulates of animal origin were tested. A more natural medium might well combine green plant and metazoan materials. Trypticase is replaceable by other commercial tryptic digests of casein. The maintenance medium is distributed in 125 x 16 mm. screw-capped tubes. One set of cultures is kept at 25–30°; another at 18–20°, in light. Cultures at 18–20° repeatedly showed many motile, viable organisms even after 8 months; those at 25–30° for about 2 months. Cultures at 6° in the dark remain alive for at least several weeks. The opalescence of the medium disappears in well-grown cultures, presumably because of the digestion of milk proteins and traces of fat.

Growth was expressed linearly as optical density, as measured with a red-sensitive photocell. A red-sensitive light probe was preferred over a blue-sensitive one. Chlorophyll has an intense absorption band in the red, hence photosynthetic organisms absorb more of the effective light. Furthermore, the blanks are reduced with yellowish or brown media. The linearity of response of the instrument was determined with permanent india ink standards, as well as by comparing dilutions of the chrysomonads themselves.

A fairly accurate indication of the trend of growth was generally obtainable in 5 to 7 days with an inoculum of 50 to 200 flagellates. Growth was substantially complete usually in about two weeks.

Cultures

Ochromonas malhamensis Pringsheim, "Pringsheim" strain. Isolated by Doctor T. Y. Chen from a mountain pond in Yorkshire; sent by Doctor Pringsheim.

O. malhamensis, "variabilis" strain. Isolated by L. Provasoli from a crude culture sent by Doctor Harley P. Brown, University of Oklahoma.

O. malhamensis, "strain No. 2". Isolated by L. Provasoli from pond water.

Poteriochromonas stipitata Scherffel. Isolated from a freshwater + soil culture by Doctor R. A. Lewin, then at Yale University.

As none of these organisms grow on stiff agar, they were isolated by the washing technique. All cultures used were clonal. They may be obtained in England from the Culture Collection of Algae and Protozoa, the Botany School, Downing St., Cambridge.

Results

The Pringsheim strain of *O. malhamensis* and *P. stipitata* were studied in greatest detail because they generally grew more vigorously than did the other two strains.

The following chemically defined medium (TABLE 1) supports heavy growth (optical density >6.0) of the 4 chrysomonads. It epitomizes the findings on the minimal requirements and embodies a partial identification of sparing and stimulatory factors.

Substrates. For studies of substrates and their interrelations with growth factors, the following basal medium ("EDTA base") was developed:

EDTA*	0.05 g.	thiamine HCl	0.2 mg.
$CaCO_3$	0.015 g.	biotin	0.5 μg.
$MgSO_4 \cdot 7H_2O$	0.06 g.	cobalamin	0.4 μg.
KH_2PO_4	0.03 g.	NH_4HCO_3	0.04 g.
DL-Methionine	0.04 g.		
Metals 42†	1.2 ml.		
Mo (as NH_4 salt)	2.0 mg.		

H_2O to 100 ml. Heat to dissolve and to drive off CO_2. Final pH 4.9–5.3.

* Ethylenediamine tetraacetic acid. It is not metabolized.

† Metals 42″ has the following composition (mg./ml.):

EDTA	2.5	Fe	0.1
Zn	4.0	Boron	0.1
Mn	3.0	Co	0.04
Cu	0.25		

This metal supplement, like that previously described containing citric acid, is unstable in light. The same salts were used as in "Chrysomonad Metals No. 2".

The pattern of substrate requirements would be more clearly understood, but for the fact that many nutrients, *e.g.*, histidine and citrate, play more than one role. There appears to be two interrelated requirements, both absolute: (1) sugar (or glycerol); and (2) any one of several citric acid cycle components. To illustrate these complications, histidine at first appeared to be an absolute requirement, but in several later experiments in which an excess of ammonium ion, citric acid, cobalamin, methionine, and glutamic acid were supplied together, there was an appreciable, at times heavy, growth without histidine. Histidine-free media support somewhat better growth in the dark, which suggests that the CO_2 concentration may affect this requirement. In the light, photosynthesis should reduce the CO_2 concentration. Growth in the EDTA basal medium was improved by citrate or succinate, but these experiments were based on media containing an excess of histidine and glutamic acid. These may have masked an absolute requirement for a citric acid cycle component. Histidine and glutamate, in most organisms, are readily brought into the citric acid cycle. Experiments aimed at clarifying this situation are in progress and a detailed discussion is best left for a later communication. An experiment in which the EDTA basal medium was used, showing the ostensible independence of the sugar-glycerol and histidine requirements, is outlined in TABLE 2.

In no experiment, was it possible to eliminate simultaneously sugar and glycerol—not even in light and in the presence of excess citric acid, histidine, and glutamic acid. Several experiments were performed to determine to what extent light spared this sugar-glycerol requirement. Little if any sparing was observed. The need for sugar or glycerol was manifest also in the presence of high concentrations of amino acids. One such experiment is shown in TABLE 3. In another section of this experiment too lengthy to summarize in TABLE 3, there was no growth with various concentrations of acid-hydrolyzed gelatin or a complete mixture of amino acids in the absence of sugar and glycerol.

Cobalamin. This requirement in chrysomonads appears to be more specific than in most other cobalamin-dependent organisms. Indeed, the pattern of specificity seems, so far, to be virtually identical with that of birds and mammals.[15] The application of one of these chrysomonads to the assay of cobalamin is described in another paper.[1] As mentioned in the discussion of this paper, another cobalamin-requiring phagotrophic flagellate, *Peranema*,[13] is under study, and several planktonic flagellates, including chrysomonads, require cobalamin.[10] It seems advisable, therefore, to defer the detailed exposition of the specificity of the cobalamin requirement until comparative data are available and limit ourselves to a brief summary of the present information.

TABLE 1

COMPLETE MEDIUM

$(NH_4)_2H$ citrate	0.12 g.	Thiamine HCl	0.2 mg.
$CaCO_3$	0.015 g.	Biotin	0.4 μg.
$MgCO_3$ (basic)	0.05 g.	Cobalamin	0.5 μg.
L-Glutamic acid	0.3 g.	Chrysomonad Metals No. 2	1.0 ml.
KH_2PO_4	0.03 g.	Mo (as NH_4 salt)	2.0 mg.
DL-Methionine	0.06 g.	NH_4HCO_3	0.04 g.
L-Histidine HCl·H_2O	0.05 g.		
L-Arginine HCl	0.04 g.	Dist. water to 100 ml.	
Sucrose	1.2 g.	pH 4.9–5.3	

Notes on Table 1.

This medium can be made up double strength and refrigerated without precipitation.

1. "Chrysomonad Metals No. 2". 1 ml. =
citric acid·H_2O 3.0 mg.
Zn 4.0 mg. (as $ZnSO_4 \cdot 7H_2O$; factor 4.4)
Mn 4.0 mg. (as $MnSO_4 \cdot H_2O$; factor 3.076)
Fe 0.2 mg. as [$FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$; factor 7.02]
Cu 0.1 mg. (as $CuSO_4 \cdot 5H_2O$; factor 3.94)
Co 0.05 mg. (as $CoSO_4 \cdot 7H_2O$; factor 4.77)

This concentrated solution is somewhat light-sensitive as shown by a progressive deepening of color in light but, if protected, keeps for months. The salts were chosen for their constancy of composition at varying humidities. It was convenient to supply the Cu and Co in this solution from concentrated stock solutions (1.0 ml. = 100 mg.). The citric acid combines the function of metal-solubilizer, metal buffer, and helps satisfy a citric acid cycle component requirement.

2. $MgCO_3$ (Basic). Because of the excess acidity introduced by the glutamic acid, it was convenient to supply Mg and Ca as carbonates, thus achieving a partial neutralization and avoiding ballasting the medium with excess anions. The basic $MgCO_3$ was designated as "$3MgCO_3 \cdot Mg(OH)_2 \cdot 3H_2O$" (Fisher Scientific Co.).

3. Mo. Clear-cut molybdenum deficiencies were not observed, but as they had appeared in work with other organisms grown at high pH's, Mo was included for the sake of completeness. Mo salts are poorly soluble in acid solutions and therefore were not combined with the other metals in one stock solution. To avoid precipitation, the Mo stock solutions were adjusted to pH 8.0–8.5 with NH_4HCO_3 or KOH. Mo was supplied as $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$; in studies of NH_4 utilization $Na_2MoO_4 \cdot 2H_2O$ was used, similarly adjusted to pH 8.0–8.5.

4. $(NH_4)H$ Citrate. Both ammonia N and citrate are readily utilized, but at the pH of this medium rather high levels are needed for dense growth.

TABLE 2

EFFECT OF HISTIDINE, SUCROSE, AND GLYCEROL ON GROWTH IN LIGHT AND DARKNESS (PRINGSHEIM STRAIN; EDTA MEDIUM)

	Light	Dark
(1) No addition	0	0.02
(2) Sucrose 0.8%	0	0.44
(3) " " * L-histidine 0.04%	3.25	2.60
(4) " " " " + Na H glutamate 0.3%	2.50	2.70
(5) Na H glutamate 0.2% + L-Glutamic acid 0.2%	0.01	0.04
(6) Medium "5" + glycerol 0.6%	0.01	0.03
(7) " " + sucrose 0.8%	0.13	0.65
(8) " " + glycerol 0.6% + L-histidine 0.04%	4.0	0.67

*"L-Histidine" = L-histidine HCl·H_2O. The weight shown refers to the hydrated salt.

The only compound sparing cobalamin is methionine. Maximal sparing is exerted at DL-methionine concentrations as high as 0.08–0.1 per cent. Methionine, in turn, is not spared by choline (or betaine) + homocystine. The highest concentrations tried were choline HCl 3.0 mg. per cent and homocystine 5.0 mg. per cent. The saturation levels for methionine appear unphysiologically high and a concentration of 0.04 per cent was recommended for the assay medium. It was considered unlikely, in view of the sensitivity of the organism to cobalamin, that the methionine in natural materials would seriously interfere. Sparing by cystine is a possibility, but concentrations of cystine above 0.015 per cent were toxic. Below this no sparing was observed. Recent experiments indicate that cystine toxicity is lessened by certain amino acids and so this phase of the investigation is to be re-opened. The cobalamin activity of commercial yeast autolysate was negligible, as was also that of plant products such as grass juice (cerophyll), and orange and tomato juice. This points to desoxyribosides being inactive. Commercial desoxyribosenucleic acid had no effect on the response to cobalamin. Treatment of 15-unit liver with alkali (pH 10.5–11) for 20 minutes at 118–121°C. completely destroyed the cobalamin activity.

TABLE 3

EFFECT OF AMINO ACIDS ON THE SUGAR-GLYCEROL REQUIREMENT (EDTA MEDIUM SUPPLEMENTED WITH L-HISTIDINE HCL·H_2O + L-GLUTAMIC ACID 0.2%; PRINGSHEIM STRAIN)

		A*
(1) No addition	0.02	0.03
(2) Glycerol 0.1%	0.72	0.80
(3) Glycerol 0.25%	1.34	1.45
(4) Glycerol 0.8%	3.6	3.1
(5) Sucrose 0.1%	0.56	0.66
(6) Sucrose 0.25%	1.12	1.25
(7) Sucrose 1.0%	2.35	3.25

* A = DL-alanine 0.05%
+ L-arginine HCl 0.06%
+ L-asparagine·H_2O 0.05%
+ glycine 0.04%
+ DL-serine 0.04%

Discussion

The 4 strains, isolated from widely separate sites, all require cobalamin and thiamine. Whether the "No. 2" and "variabilis" strains need biotin has not yet been determined. If the planktonic freshwater and marine chrysomonads now in pure culture (*Synura* sp. and 2 coccolithophorids respectively)[10] should all need cobalamin and perhaps thiamine also, then it is not a remote possibility that these vitamins may be needed by all chrysomonads. Much the same situation holds for the euglenids. The ecological and phylogenetic implications of auxotrophy in algae are discussed elsewhere.[10] It is hoped that these fresh-water chrysomonads will provide a useful point of departure for working out the requirements of those marine forms which have lately been recognized as being of great importance in the economy of the ocean. Knight-Jones and Walne[7] found that the waters around the British Isles contained as many as 1000/ml. of a minute (1.5 μ) pigmented chrysomonad; and Imai and coworkers[5] use a colorless chrysomonad as food for larvae of oysters and other marine animals in artificial culture. This chrysomonad is abundant in a bay supporting a vigorous shellfish population.[6]

Morphology of Poteriochromonas. When first isolated the organism rested in a goblet or lorica—its generic distinction from *Ochromonas*. A few individuals in the culture originally received possessed a lorica. It has been absent in the subcultures. Professor Pringsheim informs us that under certain conditions the lorica may reappear. The utility of pure culture studies in evaluating taxonomic criteria is thus illustrated again.

Requirement for a citric acid cycle component. A central question in the metabolism of any phagotroph is the readiness with which the different kinds of foodstuffs are digested and interconverted into fuel and building blocks. Since these chrysomonads consume fats, proteins, and carbohydrates, they are favorable objects for studying these interrelations. Their photosynthesis adds an extra fillip. In animals and many protists, the interconvertibility of foodstuffs is mediated *via* the citric acid cycle (CAC). Should these chrysomonads genuinely need a CAC intermediate, then they might serve as reagents for tracing links between the CAC and other reaction chains.

These chrysomonads have the simplest nutritional requirements of any animal hitherto studied. This, coupled with their omnivorousness and retention of photosynthesis, makes it tempting to speculate which of their features are common to all animals and which are peculiar to the species and the phylum. Their CAC requirement elicits the question of whether other animals have a similar requirement. The CO_2 requirement of many heterotrophic organisms, including mammals, points to a need for a CAC intermediate, since experiments with radioactive tracers indicate that most of the CO_2 is taken up in the 4-carbon dicarboxylic acids of the CAC.[14] Additional support for the view that the need for a CAC intermediate underlies the CO_2 requirement may be adduced from the experiments of Bovet *et al.*,[2] in which the respiratory center of anesthetized dogs was stimulated equally well by succinate, fumarate, or CO_2. One reason that the status of a CAC requirement in animals remains doubtful is that the required CAC intermediate might be produced from an excess of amino acids and carbohydrate. One necessary step was taken only recently: When birds and mammals are fed adequate but not excessive amounts of essential amino acids, there is a net uptake of ammonia N. It would be interesting to see whether administration of one or more CAC intermediates might spare the requirements for amino acids. *Tetrahymena pyriformis* might be a convenient test organism now that it can be grown in synthetic media. The thiamine and biotin requirements of these chrysomonads provide additional avenues of approach to the reactions of the main metabolic pool.

Cobalamin. As previously noted, the specificity of this requirement in chrysomonads approaches most closely that shown by vertebrates. The limited response to various pseudo-B_{12}'s (cobalamin-like compounds which have cobalamin activity for various microorganisms but not for animals)[1] supports this idea. The demonstration of a cobalamin requirement in *Peranema*[10] creates the opportunity to compare the patterns of cobalamin specificity and availability of two phagotrophs as remote phylogenetically from each other perhaps as both are from metazoa. A cobalamin requirement may not hold for all animals, unlike biotin and thiamine requirements, since insects have not been reported to need cobalamin. Exploration of the ability of chrysomonads and of *Peranema* to utilize cobalamin in high-molecular combination is but another facet of the general problem, as yet hardly more than defined, of the factors governing the utilization of particulate food by phagotrophic cells and protists. Some potentialities of phagotrophic protozoa as reagents for high-molecular compounds are sketched elsewhere.[4]

Glucose-glycerol. As yet, glycerol is the only compound that can replace sugars and still allow heavy growth. The readiness with which these organisms accumulate oil (old cultures, incidentally, have an odor resembling slightly rancid olive oil) leads to the supposition that one of the principal functions of sugar is to provide glycerol for the esterification of fatty acids, as has been postulated for the mammary gland.[3, 8] The faster growth achieved with glucose or sucrose indicates, as is to be expected, that glucose functions in additional capacities.

Concluding Remarks. Each aspect of the biochemistry of chrysomonads touched on here warrants a detailed investigation. Adequately to define their phagotrophic capacities would alone be a vast undertaking. As the greater part of the results mentioned in the present paper were obtained under the widely different experimental conditions necessary in the development of reproducible chemically defined media, it seemed prudent not to attempt more than a preliminary brief piecing together of relevant experiments. Nevertheless, these flagellates, although newcomers to the biochemical scene, have already been put to biochemical and morphogenetic use, as shown by several papers from other laboratories included in this monograph.

Summary

The minimal nutritional requirements of four fresh-water phagotrophic photosynthetic chrysomonads include cobalamin, thiamin, biotin (for at least two strains), carbohydrate (or glycerol), and possibly a citric acid cycle intermediate. Involved in this latter requirement are histidine, citric and glutamic acids.

Cobalamin was spared by methionine. Methionine was not spared by choline + homocystine.

The minimal requirements were the same in light and darkness.

These chrysomonads are deemed to have the simplest exogenous requirements of any animals hitherto studied, and are recommended for studies of the biochemical systems underlying the animal mode of life.

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PRELIMINARY STUDIES OF THE RESPONSE OF A CHRYSOMONAD TO VITAMIN B_{12} AND RELATED SUBSTANCES*

By F. W. Barber, D. L. Baile, C. B. Troescher, and C. N. Huhtanen
National Dairy Research Laboratories, Inc., Oakdale, Long Island, N. Y.

During the Spring of 1952, S. H. Hutner submitted a culture of *Poteriochromonas stipitata* to the microbiological assay laboratory of National Diary Research Laboratory for evaluation as a vitamin B_{12} assay test organism. Our laboratory had been routinely running vitamin B_{12} assays for two years, using the *Lactobacillus leichmannii* method of Skeggs, Nepple, *et al.* Along with other workers, we felt that *L. leichmannii* responded to other substances in addition to true vitamin B_{12} and, hence, we were anxious to obtain a test organism that would respond to pure vitamin B_{12} only.

It was proposed that a comparative assay study be made on various samples, comparing the chrysomonad with other methods of vitamin B_{12} assay.

Since April of this year (1952), many vitamin B_{12} assays have been run using the chrysomonad *P. stipitata.* This paper will give assay results on various products using the chrysomonad method as compared with other methods of assay.

Methods

The chrysomonad assay was run according to the method of S. H. Hutner.[1] Minor changes were made in the basal medium, such as substituting the ammonium citrate salt with equivalent amounts of ammonium hydroxide and citric acid, substituting magnesium sulfate for magnesium carbonate, and using ammonium hydroxide rather than sodium bicarbonate to adjust the pH of the basal medium. TABLE 1 gives the composition of the basal medium.

A different stock-culture medium from that recommended was used. It consisted of basal medium supplemented with vitamin B_{12} (Cobione) and one per cent Sheffield's Peptonized Milk Nutrient. This medium sponsors heavier growth of the chrysomonad than the original stock-culture medium used.

Samples for assay were prepared in the usual manner for vitamin B_{12} assay by dissolving the material in a large volume of water and heating to 100°C. for 20 minutes for solids and viscous liquids, and by merely diluting with water for purified extracts. Each sample was diluted to contain approximately 0.0001 μg. B_{12} per ml. Samples were prepared in the same way for all methods of vitamin B_{12} assay.

The standard was prepared from crystalline vitamin B_{12} (Cobione) in a range from 0.02 to 0.5 mμg. per flask. The assay was carried out in 50 ml. Erlenmeyer flasks plugged with cotton.

The inoculum was prepared from a week-old culture of *P. stipitata* grown in a tube of culture medium at room temperature over a white fluorescent light. The culture was centrifuged for five minutes, the medium decanted, and the culture resuspended in 12 ml. of 0.9 per cent sterile saline. The assay flasks were inoculated with one drop of cell suspension per flask.

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* The authors wish to express their appreciation to Miss Mary Hulse and to Miss Grace O'Brien for their technical assistance.

The chrysomonad assay was incubated at room temperature (25–31°C.) over white fluorescent lamps for from five to seven days. The flasks were shaken once daily to stimulate growth. Growth was determined by optical density measurements read in a model 400-A Lumetron colorimeter using the blue filter no. 420.

Other vitamin B_{12} assay methods used for comparison were the *L. leichmannii*, *E. coli* mutant, and *Euglena* methods. The *L. leichmannii* method, probably the best-known and most-widely-used method of B_{12} assay, was run according to the method of Skeggs, Nepple, *et al.*,[2] using vitamin-free, salt-free acid-hydrolyzed casein* (a modified Hycase) in the basal medium, and acidimetric measurements of growth.

The *E. coli* mutant method was that of Davis,[3, 4] using a fast B_{12}–methionine mutant (no. 113-3), an 18-hour incubation method read turbidimetrically. This is a less-sensitive test organism than the others, responding in the range of 0.1–2.0 mμg. of vitamin B_{12}. Unfortunately, the *E. coli* mutant also responds to methionine, so that materials high in this amino acid give erroneously high vitamin B_{12} values on assay.

TABLE 1
CHRYSOMONAD ASSAY BASAL MEDIUM
Double Strength

Ammonium hydroxide (conc.)	0.2 ml.%
Citric acid	0.14 g.%
$CaCO_3$	0.03 "
$MgSO_4 \cdot 7H_2O$	0.1 "
KH_2PO4	0.06 "
L-Glutamic acid	0.6 "
DL-Methionine	0.12 "
L(+)histidine monohydrochloride	0.1 "
Sucrose	2.4 "
Thiamine	0.4 mg.%
Biotin	1.0 μg.%
Mo (as Na molybdate)	4.0 mg.%
Metals solution	2.0 ml.%

Heat to boiling point, cool. Adjust to pH 5.2–5.4 with NH_4OH.

The *Euglena* method[1, 5] was that of Hutner, who kindly supplied the culture of *Euglena gracilis* with the procedure. The assay technique was similar to the chrysomonad assay as to incubation conditions and handling of the inoculum.

Results and Discussion

The standard curve (FIGURE 1) shows the response of the chrysomonad to pure vitamin B_{12} in a range from no B_{12} to 0.5 mμg. B_{12} per flask in eight graduated levels. Growth turbidities were measured as optical densities. The zero level has a very low optical density, which shows the specific need of the organism for vitamin B_{12}.

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* Made by National Dairy Research Laboratories, Oakdale, N. Y.

TABLE 2 gives assay results on products assayed by four different methods. The values shown in the first column are the chrysomonad-assay values and are usually much lower than the values seen in the other columns. The numbers in the three narrow columns interspersed between the assay values are ratios of the alternate method values to the chrysomonad assay value, taking the latter as one. This system was devised merely as a quick means of ap-

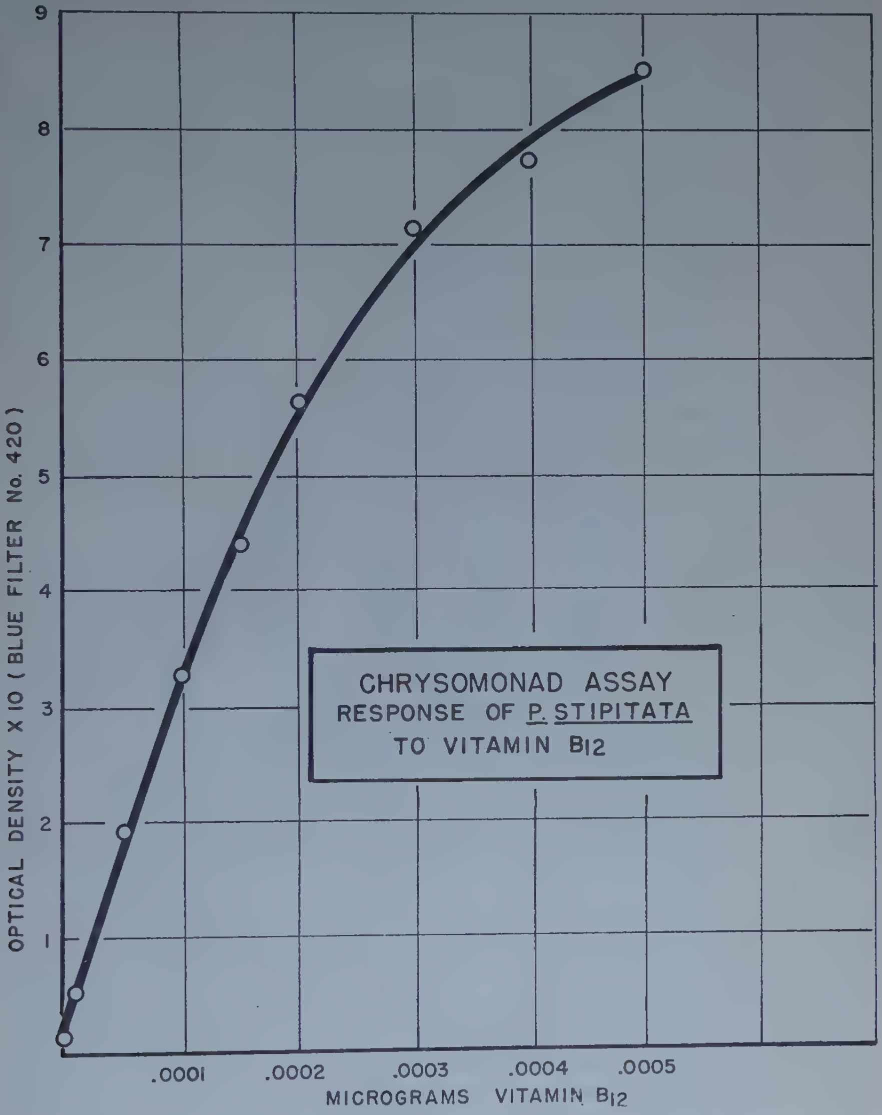

FIGURE 1. Standard curve.

praisal of the correlation between methods, a low number indicating good correlation, a high number poor correlation.

It is felt that the chrysomonad values are true vitamin B_{12} values, while the other values indicate vitamin B_{12} plus pseudovitamin B_{12}, of which there are several forms.[6, 7] Pseudovitamin B_{12} has not been demonstrated to have any activity for animals. High ratios in TABLES 2 and 3 suggest, therefore, considerable pseudovitamin B_{12} activity.

The data in TABLE 2 show that the best agreement among the four methods was on dried liver extract. This would indicate that the B_{12} activity found in liver is due to true vitamin B_{12}. Clinical evidence bears out the presence of the biologically active form of vitamin B_{12} in liver.

Bi-Con* and Anigene† are commercial animal-protein-factor supplements used in animal feeds. Although the three alternate assay values are higher than the chrysomonad, the ratios are not high (*E. coli* value is lower), which indicates that the B_{12} activity is largely due to true B_{12}, as might be expected in animal feed supplements, since true vitamin B_{12} is the biologically active form. Dried clam juice's activity is largely due to true B_{12}, as indicated by the low ratios. *E. coli* value is highest, probably due to the methionine content.

TABLE 2
COMPARATIVE ASSAY VALUES

Sample	Vitamin B_{12} micrograms per gram or ml.						
	P. stipitata	Ratio	*L. leichmannii*	Ratio	*E. coli*	Ratio	*Euglena*
Dried liver extract	0.98	1.5	1.5	1.5	1.5	1.1	1.08
Bi-Con	2.98	2.2	6.6	0.5	1.5	—	—
Anigene	8.50	2.5	21.4	2.1	17.7	1.7	14.7
Dried clam juice	2.48	2.0	5.07	2.9	7.3	1.8	4.6
Chicken droppings	0.04	15	0.61	2.4	0.096	11.5	0.46
Chicken droppings	0.05	10	0.50	1.2	0.06	9.6	0.48
Human infant feces	0.003	17	0.051	34.6	0.104	23.3	0.07
Rumen contents	0.052	7	0.37	—	—	3.7	0.192
Alfalfa juice conc.	0.0017	1130	1.91	882	1.5	376	0.64

Assay values on feces and rumen contents show a greater variation than those products already mentioned, with a ratio as high as 1:34 in one case. Lewis *et al.*[8] found in fecal matter of rats a form of vitamin B_{12} that differed in both physical and biological properties from true vitamin B_{12} having microbiological activity but little animal growth response. Alfalfa juice shows the greatest variation of any sample assayed by the four methods, with a ratio of about 1:1000 in two instances, and also high in the third. This would indicate that only a very small portion of its apparent vitamin B_{12} activity is due to true B_{12}.

TABLE 3 shows more comparative assay results on a group of related products. The first six are purified bacterial extracts. The RC samples are dried forms of similar cultures of bacteria. The chrysomonad assay values are very low, while the other assay values are considerably higher, the high ratios indicating considerable pseudovitamin B_{12} activity. This fact was borne out by feeding experiments with animals in which the RC samples showed low vitamin B_{12} biological activity in spite of high *L. leichmannii* assay.

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* Bi-Con—Chas. Pfizer & Co., Inc., Brooklyn, N. Y.
† Anigene—Schenley Distillers, Inc., Cincinnati, Ohio.

J20 and J21 are fractions of RC 105 separated by the Craig countercurrent apparatus. Unfortunately, the fractions were not pure. J20, intended to be pure true vitamin B_{12}, had some contaminating substances in it, and J21 was not completely purified pseudovitamin B_{12}. It is interesting to note, however, that the nearly pure substances gave quite different B_{12} assay spectra, with very high ratios for *L. leichmannii* and *E. coli* on J21, and low ratios on J20. The very low value obtained for J21 (the pseudo B_{12} preparation) by the chrysomonad method compared to the high values obtained by the other methods indicates that *P. stipitata* does not respond to pseudovitamin B_{12}. Recently we were fortunate enough to obtain three purified preparations of pseudovitamin B_{12} from Doctor J. J. Pfiffner of Parke-Davis and Company. The chrysomonad did not give any growth response to any concentration of the pseudovitamin B_{12} preparations tested.

TABLE 3
COMPARATIVE ASSAY VALUES

Sample	Vitamin B_{12} micrograms per gram or ml.						
	P. stipitata	Ratio	*L. leichmannii*	Ratio	*E. coli*	Ratio	*Euglena*
Rumen bact. extracts							
I	0.005	118	0.59	50	0.25	34	0.17
II	0.0045	62	0.28	45	0.20	13	0.06
III	0.0026	42	0.11	33	0.09	38	0.10
IV	0.004	90	0.36	48	0.19	—	—
V	0.004	55	0.22	—	—	—	—
Pooled sample	0.0026	142	0.37	92	0.24	30	0.079
Concentrated cells	0.153	167	25.5	65	10	4	0.65
RC 105, dried	0.03	160	4.8	323	9.7	61	1.81
RC 245 + 246, dried	0.044	375	16.5	306	13.5	46	2.0
Craig counter current fractions:							
J 20—B_{12} + cont.	0.044	32	1.41	10	0.42	1.3	0.057
J 21—Pseudo B_{12}	0.009	811	7.3	622	5.6	1.9	0.017

Reliability

The reliability of a given assay procedure can be assessed in terms of recovery and reproducibility.

Vitamin B_{12}, when mixed with crude samples and subjected to the chrysomonad assay procedure described, was available for the growth of *P. stipitata.* The percentage recovery of vitamin B_{12} is shown in TABLE 4. The average recovery of vitamin B_{12} was 105 per cent. Recoveries of 90–110 per cent are usual for microbiological assay procedures.

TABLE 5 shows the reproducibility of the chrysomonad assay procedure. The average results shown were obtained from five assays carried out by two different individuals on three different preparations of samples. The average deviation of 10.8 per cent falls within the range usually encountered in microbiological assays.

TABLE 4
RECOVERY OF VITAMIN B_{12}

Sample	Vitamin B_{12}, mμg./flask			Recovery, per cent
	Added in sample	Added crystalline	Found	
Dried clam juice	0.02	0.10	0.14	117
	0.045	0.10	0.18	
	0.072	0.10	0.21	
	0.10	0.10	0.22	
Bi-Con	0.015	0.10	0.12	97
	0.03	0.10	0.13	
	0.04	0.10	0.135	
	0.05	0.10	0.132	
Bact. extract	0.02	0.10	0.125	101
	0.023	0.10	0.123	
	0.025	0.10	0.121	
	0.027	0.10	0.130	
				Average: 105

TABLE 5
REPRODUCIBILITY OF CHRYSOMONAD VITAMIN B_{12} ASSAY PROCEDURE

Sample	Range, μg./g.	Av. vitamin B_{12},* μg./g.	Deviation, %
Dried clam juice	2.15–2.70	2.39 ± 0.22	9.2
Bi-Con	3.32–4.73	3.83 ± 0.54	14.0
Liver extract	0.54–0.71	0.65 ± 0.06	9.2
			Average: 10.8

* Average of 5 separate determinations.

Summary

(1) An assay procedure for vitamin B_{12} using the chrysomonad *P. stipitata* has been described.

(2) Comparative assay values on 20 products using four different methods of vitamin B_{12} assay have been presented.

(3) The chrysomonad assay is the only one of the four methods studied that appears to respond to true vitamin B_{12} alone. Experimental results indicate that the chrysomonad does not respond to pseudovitamin B_{12}.

(4) The reliability of the chrysomonad assay, as shown by recovery and reproducibility experiments, falls within the limits of an acceptable microbiological assay procedure.

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DISCUSSION OF THE PAPERS ON VITAMIN B_{12}*

By David Hendlin
Merck & Company, Inc., Rahway, New Jersey

The report by Hutner and coworkers in 1949 that *Euglena gracilis* responds to the antipernicious anemia factor (vitamin B_{12}) and not to desoxyribosides (thymidine) was regarded as a major step forward in the search for a microbiological assay specific for vitamin B_{12}. This was primarily because the only other vitamin B_{12}-dependent microorganisms, lactobacilli and mutant strains of *Escherichia coli*, had the disadvantage of responding to compounds physiologically related to vitamin B_{12}.

It soon became the experience in many laboratories, as has been indicated in the preceding paper from the National Dairy Laboratories, that *Euglena gracilis* itself was not specific in its requirement for vitamin B_{12}, that it too responded to substances other than vitamin B_{12}, especially substances of microbial origin. In our own laboratory we observed similar phenomena. In screening for vitamin B_{12} producers among isolates from cow and sheep rumen contents, manures and organisms from our culture collection, we very early in the program came across anaerobes, facultative and obligate, that synthesized factors capable of supporting growth of *Euglena gracilis* and which by paper chromatography and chemical fractionation did not appear to be vitamin B_{12} or related cobalamins. One such organism *Clostridium tetanomorphum* yielded broth cultures which were active for *Lactobacillus lactis*, *Lactobacillus leichmannii*, *Escherichia coli* and *Euglena gracilis* but possessed no significant biological activity in replacing vitamin B_{12} in the rat or the chick. Mrs. Baile's data on pseudovitamin B_{12} indicates that clostridium factor could not be pseudovitamin B_{12} since in our hands the clostridium factor has equal activity for *Euglena gracilis* and the lactobacilli.

In an attempt to learn something of the nature of the B_{12} replacing factors, study was made of two compounds considered to be chemical precursors of vitamin B_{12}, 1,2-dimethyl,4,5-diaminobenzene and 5,6-dimethylbenzimidazole. These compounds as well as their ribityl analogs were found to be devoid of vitamin B_{12} activity. More than that the dimethyldiamine and its ribityl analog as well as the dimethylbenzimidazole were found to be inhibitory to *Euglena gracilis*. The inhibition of the dimethyldiamines was antagonized by vitamin B_{12} (TABLE 1).

It is clear from the papers presented today as well as our reported data that *Euglena gracilis* like the lactobacilli and vitamin B_{12}-dependent strains of *E. coli* is not specific in its requirement for vitamin B_{12}. In the light of these experiences, data such as those reported by Doctor Robbins cannot be interpreted as indicating the true vitamin B_{12} content of natural substances. Perhaps the chrysomonad assay is the answer to the search for a microbiological assay specific for vitamin B_{12}. Further and widespread experience with this protozoan assay will throw some light on its specificity for vitamin B_{12}.

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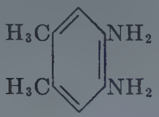
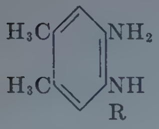
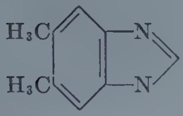
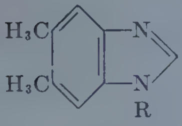
* The unpublished data presented in this discussion were obtained in collaboration with my colleagures, Misses Margaret H. Soars and Myrle L. Ruger.

TABLE 1
THE INHIBITORY EFFECTS OF B_{12} PRECURSORS

Compound	Half maximal inhibition µg./ml.	Reversal with B_{12}
	90	+
	100	+
	270	±
	>1000	

Vitamin B_{12} content of the medium was 16 µug per ml.
R = ribityl.

Discussion of the Paper

B. S. SCHWEIGERT, *American Meat Institute Foundation and Department of Biochemistry, University of Chicago:* We have been working for some time on improving methods for estimating vitamin B_{12} in animal tissues and have compared the results obtained with different methods, particularly the rat growth assay and microbiological (*Lactobacillus leichmannii*) assay. Of particular interest are the results obtained for five different assay methods on a sample of lyophilized, defatted beef liver.

Sample	Assay Method*				
	Rat Growth	Chick Growth	*L. leichmannii*	*E. gracilis*	*E. coli* Mutant
		µg. per gram			
Lyophilized, defatted beef liver	more than 18	5.0	3.5	2.6	2.1

* The chick growth and *E. coli* mutant assays were kindly performed by Doctor T. H. Jukes and Doctor W. L. Williams of Lederle Laboratories Division, American Cyanamid Company.

It can be seen that all methods agree quite well except the rat growth assay which gave much higher results. The results for certain samples, however (particularly muscle cuts), were in good agreement with the rat and microbiological assays. These studies, conducted in collaboration with Harold E.

Scheid and Mona M. Andrews, have been published recently (1952. J. Nutrition. **47:** 601). We believe that additional studies with these and other methods (*i.e.*, with other test organisms, chemical or isotope dilution methods) will be very useful, and should provide necessary information needed before a completely satisfactory method is developed for measuring the potency of vitamin B_{12} (cyanocobalamin) and related compounds in the free and bound form in a variety of natural products.

AN ELECTRON MICROSCOPE STUDY OF TWO FLAGELLATES. CHLOROPLAST STRUCTURE AND VARIATION

By J. J. Wolken*† and G. E. Palade
Laboratories of The Rockefeller Institute for Medical Research, New York, N. Y.

The advantages of cultured protozoa as a material for growth and nutrition studies are well known and have recently been stressed by Lwoff,[1] Hutner and Provasoli,[2] and Kidder.[3] Few investigations of cultured protists, however, have tried to correlate spontaneous or induced metabolic changes with structural alterations, although the material appears particularly suitable for such studies. It would be interesting, for instance, to know what happens to the chloroplasts which exist in some flagellates, when these protozoa are adapted to life in the dark. As is well known,[4-6] dark-adaptation is accompanied by the disappearance of the green pigment, a fact that undoubtedly results in important modifications in the metabolic pattern of these flagellates. This problem has already been investigated by light microscopy with the conclusion that the chloroplasts disintegrate during dark-adaptation and reform when the flagellates are brought back to light. Partly because of the limited resolving power of the light microscope, however, it has not been possible to determine whether the old chloroplasts disappear completely, or how the new ones develop.

The present work represents an attempt to investigate this problem by using the high resolving power of the electron microscope and by following, in parallel, the development of the green pigments and the structure of the chloroplasts during light- or dark-adaptation. The study is still far from complete. Accordingly, only preliminary observations will be presented, most of them concerning the morphological background of the problem; *i.e.*, the organization of the cytoplasm, chloroplasts included, as revealed by the electron microscope in the two algal flagellates we selected for our experiments.

Material and Methods

Material. These flagellates, namely *Euglena gracilis* var. *bacillaris*, and *Poteriochromonas stipitata*, a phagotrophic chrysomonad, proved to be very appropriate materials for experimental study due to the comparative ease of their handling, their fairly rapid growth, and the possibility of controlling the chemical and physical environment affecting their morphology and growth. These organisms can be grown in a chemically defined medium either in light, where they develop green pigment (chlorophyll) or in darkness, where they become colorless. The process is reversible, but can be made irreversible by heat[7] or streptomycin treatment.[7, 8]

Culture methods. Both flagellates were obtained from the Haskins Laboratories‡ and grown in the culture media reported by Hutner *et al.*[9, 10] The organisms were grown at room temperature (∼25°C.) either under continuous light (~400 foot-candles) or in complete darkness. They were cultured in cotton-plugged, 1-liter bottles containing 330 ml. fluid, or in 30-ml. test tubes containing 10 ml. fluid. No stirring or additional aeration was used. In the light, the flagellates became heavily pigmented, green for *Euglena*, and brownish-green for *Poteriochromonas*, yielding approximately 8–10 grams wet-weight of organisms per liter of nutrient fluid after 7 days of growth. When grown in darkness, their yields were smaller and the packed organisms had a creamy-white color. For *Euglena*, our yields were in agreement with the growth rates indicated by Myers.[11]

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* Present address: Biophysics Research Laboratory, Eye and Ear Hospital, University of Pittsburgh Medical School, Pittsburgh, Pa.

† This study was made while a Fellow of the American Cancer Society on recommendation of the Committee on Growth of the National Research Council.

‡ We are indebted to Doctors S. H. Hutner and L. Provasoli of the Haskins Laboratories, N. Y., who provided us with the initial cultures of the two flagellates, and advised us on culturing methods.

Light Microscopy. During growth, the organisms were examined as to their viability and general appearance with the light, phase-contrast, and polarizing microscopes. In the light microscope, *Euglena gracilis* appears as an elongated organism measuring ~70 x 20 μ, with a differentiated anterior pole where one finds the cytopharynx or gullet, out of which protrudes the flagellum. The protozoon has a limiting membrane, provided with barely visible ridges, and contains a single nucleus, 8 to 12 chloroplasts, numerous mitochondria, and a number of vacuoles and lipid inclusions. Among the latter, one finds a differentiated group, the stigma or eye-spot, located close to the gullet and containing a red pigment. This is the appearance of the actively swimming *Euglenas*, but in cultures one frequently encounters forms that are rounded-up (~35 x 25 μ) and less pigmented. Such forms, known as inactive forms, possess less numerous organelles and are more sluggish in their movements, if not completely inert. In cultures grown in darkness one encounters both active and inactive forms, but the latter predominate. All organisms seem to have lost their chloroplasts. *Poteriochromonas stipitata* is a much smaller flagellate with an almost spherical body measuring ~7 x 8 μ in diameter. It has two flagella, a smooth membrane, a single nucleus, and only a few (generally two) chloroplasts.

Electron Microscopy. For electron microscopy the flagellates were fixed in 1 per cent osmium tetroxide. Good results were obtained when the pH of the fixative was maintained at neutral or alkaline pH (7.0 to 8.0) by buffering with acetate-veronal.[12] Many organisms had a tendency to swell during fixation or post-fixation treatment. This could be prevented, at least partially, by increasing the tonicity of the fixative with added sucrose (0.15 *M*. The fixed specimens were embedded in *n*-butyl methacrylate and sectioned at 0.1 μ or less. The sections were examined in an RCA, model EMU, electron microscope, without removing the plastic.

Observations and Discussion

Our studies deal mainly with three cytoplasmic organelles; namely, the chloroplasts, the mitochondria and the endoplasmic reticulum. However, supplementary information will be presented in relation to other cytoplasmic structures such as the stigma, the flagella, and the pellicle or exoskeleton.

1. *Chloroplasts*

Morphology. The chloroplasts appear in the light microscope as elongated, green bodies, more or less cylindrical in shape and measuring ~5–10 x ~0.5–1.5

μ. In the phase contrast microscope, they show occasionally a faint lamination and in the polarizing microscope they appear to be birefringent. Both these facts, particularly the latter, suggest the existence of an orderly, laminated structure below the limit of visibility of the light microscope. In fact, a "submicroscopic" (by reference to the light microscope) laminated structure has been postulated repeatedly for the chloroplasts in general, on account of their birefringence.[13] Such a structure is demonstrated successfully in PLATE 1, FIGURES 1 and 2. FIGURE 1 shows at a relatively low magnification (7500 X) a large area of a section through an active form of *Euglena* in which the profiles of four chloroplasts can be found. Part of a section through a single chloroplast is shown at a higher magnification (30,000 X) in FIGURE 2, where the elongated organelle appears to be made up of a pile of lamellae more or less regularly spaced. Each lamella shows as a continuous, dense and homogeneous band of nearly uniform thickness. Measurements of these bands in various electron micrographs give an average thickness of 25 mμ with variations from 18 to 32 mμ. From the examination of a large number of electron micrographs, it appears that the apparent thickness varies according to the incidence of the section, the thickness increasing as the angle of cutting decreases. For this reason, we believe that the smaller figures (18 mμ) are closer to reality.

The interlamellar spaces are 30 to 50 mμ wide and contain a light and, in general, homogeneous material, although occasionally one encounters dense, spherical granules embedded in it. The spacing of the lamellae in our preparations appears to be affected by several factors, the most obvious being the fixation. At acid pH's, for instance, the lamellae seem to pull apart so that large empty spaces are frequently found between them (PLATE 1, FIGURE 1). This does not necessarily mean, however, that any and all irregularities in spacing are artifacts. The faint lamination shown occasionally by the chloroplasts of living *Euglenas* in the phase contrast microscope may in fact be due to some spacing irregularity occurring *in vivo*. The lamellae themselves are too thin and too closely packed to be resolved by light, but groups of them separated by large enough spaces may impart to the organelles the laminated appearance we are referring to. In this regard also, it should be mentioned that, in the chloroplasts of inactive forms, the piling of the lamellae remains irregular, regardless of the precautions taken during fixation. In such *Euglenas*, the lamellae appear distorted (PLATE 1, FIGURE 3) and very frequently one finds them forming curious patterns in which they appear to converge in a central granule. It may be noticed that, in such chloroplasts, the dense, spherical granules found in between the lamellae are more numerous and more variable in size (FIGURE 3). From such observations, one may be tempted to associate the regularity in piling with a high degree of chloroplast efficiency, and the decrease in regularity with low activity.

In the elongated chloroplasts of active *Euglenas*, one frequently encounters a dense central region in which the lamellae seem to be tightly held together (FIGURE 1). In some cases, they can be followed through, but in numerous others, all trace of lamination disappears from this central region, which we identify tentatively with the pyrenoid. Very frequently, the pyrenoid protrudes at the surface of the chloroplast and its protrusion is surrounded by a vacuole.

Finally, each chloroplast appears to be limited by a membrane that is difficult to distinguish from the lamellae when the latter are normally cut, but which shows up better when the lamellae are cut very obliquely and therefore seem less clearly outlined (PLATE 1, FIGURE 4).

The chloroplast of *Poteriochromonas stipitata* (PLATE 3, FIGURE 11) is built up according to the same general pattern but shows a few differences. The organism usually has only two chloroplasts flanking the nucleus and connected by a sort of bridle that goes over the anterior pole of the nucleus. The lamellae are fewer and thicker than in *Euglena*. They measure approximately 30 mμ, and, perhaps because of this increased thickness, it is possible to resolve some indication of internal structure. Each lamella appears to have a central layer of lighter material covered on both sides by thinner and denser layers.

Some of these morphological data have been reported in an earlier publication.[14] The chloroplasts of these flagellates seem to have the same fundamental structure as the chloroplasts of some recently studied algae.[15]

Dark ⇆ Light-Adaptation. In flagellates which have become colorless after being grown in darkness for periods longer than 7 days, there is an obvious change of appearance in relation to the chloroplasts. They seem to vanish beyond recognition in *Euglena* and, while in *Poteriochromonas* the paranuclear chloroplasts may still be recognized, they are shrunken and their lamination appears to be partly or totally lost.

Such dark-adapted, colorless *Euglenas* were fixed at regular time intervals after having been brought back to light and it was found that, after as little as 4 hours light exposure, elongated bodies with the characteristic lamination of chloroplasts began to show in the thin sections. At the beginning, their lamellae were thinner, much less numerous, and much less tightly and regularly packed than in the usual light-adapted flagellate. Thereafter, the lamellae increased progressively and continuously in number so that, after 72 hours light exposure, the chloroplasts had almost the form and organization described above for active *Euglenas* grown in light for generations. The development of chloroplasts is accordingly a very rapid process. Unfortunately, what has thus far been seen in colorless *Euglenas* exposed to light for short intervals does not permit us to reach definite conclusions concerning the origin of the apparently new chloroplasts. In any case, we found no evidence of the transformation of mitochondria into chloroplasts as sometimes advocated by plant cytologists.[16]

Spectroscopic Studies. The profound changes undergone by the chloroplasts during the process of dark- or light-adaptation have an obvious chemical counterpart, namely: chlorophyll seems to disappear entirely when the flagellates are grown in darkness, and appears to be rapidly synthesized while the organisms re-adapt themselves to light. Chlorophyll variations can, of course, be studied quantitatively during the process and such a study can be correlated with the morphological changes observed. By so doing, it may be hoped that our understanding of the entire problem will be improved.

In the experiments to be described, *Euglena* cultures were put through the process of adaptation either to darkness or to light. Aliquots were taken therefrom at regular time intervals, extracted with 80 per cent acetone or with methanol, and the absorption curves of the various extracts recorded in a Carey spectrophotometer.* Such extracts contain, besides chlorophylls, other pigments, mostly carotenoids, which do not necessarily come from the chloroplasts. In FIGURE 6, the absorption curve marked 0 hrs. is that of an extract obtained from *Euglenas* cultured in light for generations. On this curve, the various peaks can be identified as belonging to chlorophylls and carotenoids. The peaks around 630 and 660 mμ, for instance, are due to chlorophyll b and a, respectively,[17] while those between 400 and 500 mμ are produced jointly by carotenoids and chlorophylls. The curve marked 0 in FIGURE 5 corresponds to the dark-adapted *Euglenas*, and indicates that although the protists look colorless, they still contain a small amount of chlorophyll and presumably a slightly larger one of carotenoids. The curves marked 3, 6, 11, 24, and 48 hours show that, when the dark-adapted organisms are exposed to light, the amount of pigment decreases during the first few hours, a fact which could have some relation to the bleaching of chlorophyll induced by light *in vitro*. After 11 hours, the synthesis overcomes the bleaching and, between 24 and 48 hours, a rapid increase in chlorophyll occurs. FIGURE 6 illustrates the fate of the pigments in the reversed experiment. The disappearance of chlorophyll seems to be more gradual than its formation, and measurable amounts of chlorophyll appear to persist after 3 days of growth in darkness.

In these adaptation experiments, our data, both morphological and spectrophotometric, are quite limited, so that no definite conclusions can be expected from them. If, however, one tries to correlate them in order to give direction to further work, the following two facts are of possible significance: (1) Some chlorophyll persists in colorless *Euglenas* which apparently have lost their chloroplasts. It may possibly be that chloroplast remnants containing this chlorophyll persist also, although we are not yet able to identify them as such in *Euglena*. As mentioned above, recognizable chloroplast remnants persist in *Poteriochromonas*. (2) There is no clear parallelism between chlorophyll synthesis and formation of chloroplast lamellae, at least during the first hours of re-adaptation to light.

2. *Mitochondria*

The presence of mitochondria and their general disposition in *Euglena* has been studied by Hovasse,[6] who found that this flagellate has a voluminous chondriome and that very frequently its mitochondria are organized in reticula.

From our electron micrographs (PLATE 2, FIGURE 7) it appears that mitochondria have a limiting membrane, 7–10 mμ in thickness, and a system of internal ridges that protrudes from the inside surface of this membrane towards the interior of the organelles. The ridges are, in general, perpendicular to the long axis of the mitochondria, but occasionally one finds ridges out of alignment, and then one sees that they are like flaps with a triangular or rounded profile. The ridges (cristae mitochondriales) do not reach the other side of the organelle, so that between series of cristae protruding from various sides there is usually a central channel left free. The space bounded by the mitochondrial membrane is filled by a light matrix, presumably fluid, *in vivo*. It is in this fluid matrix that the cristae mitochondriales are bathing in the living state. All these features have been described for mitochondria of higher animals,[18, 19] and it is interesting to point out that the same pattern of mitochondrial organization prevails throughout the animal kingdom, from protists up to mammals. One of us has already discussed the implications of this type of organization and has pointed out some facts which suggest that the oxidative enzymes of the mitochondria are built in these cristae mitochondriales.[19]

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* We are indebted to Doctor H. Jaffe of the Rockefeller Institute for Medical Research, who helped us in the recording of the absorption curves.

A similar pattern is encountered in the mitochondria of *Poteriochromonas*, although here the "cristae" appear to be less rigid, rather flexible, and less regularly distributed (PLATE 3, FIGURE 13).

In the dark-adapted *Euglenas*, there is a considerable increase in the number of mitochondrial profiles one finds in the sections, but the organization pattern is unchanged. In such flagellates, most of the organelles are "marginated," *i.e.*, one finds most of them lying immediately underneath the pellicle (PLATE 2, FIGURE 7). In the light-adapted organisms, the distribution is less preferential, but a good number of organelles are found around the chloroplasts. This might indicate that the local oxygen tension is one of the factors determining the position of mitochondria in the cell.

3. *Endoplasmic Reticulum*

Both chloroplasts and mitochondria represent organelles well-known and much studied in the light microscope. In their case, the electron microscope has contributed only more details to fit in an already outlined frame. The situation is quite different for the endoplasmic reticulum. This is a complex cytoplasmic system of vesicles and tubules, first revealed by the electron microscope in animal cells cultured *in vitro*. In such material, it has been studied extensively by Porter.[20] More recently, vesicles and tubules of the same size and electron density have been described by way of thin sections in practically all cells *in situ*, by Palade and Porter,[21] who proposed that this material is responsible for the basophilia of the cytoplasm and that it corresponds to the microsomes isolated by differential centrifugation from tissue homogenates, as first done by Claude.

It appears that the elements of this system may be integrated in a continuous network in the endoplasm of the cell. In such cases, it deserves, naturally, the name of endoplasmic reticulum. In other cases, however, these elements are vesicular, independent, and more or less evenly scattered throughout the cytoplasm. From more recent studies, it appears that, in active cells, the tubular elements are dominant and that they are frequently closely packed together in bundles or skeins. *Euglena*, like many other animal cells, appears to possess both bundles of tubular elements and a scattering of vesicular elements in its cytoplasm, as shown in PLATE 2, FIGURE 8. Similar features are encountered in *Poteriochromonas* and, in this respect, there is no difference to be noted between the dark- and light-adapted flagellates.

4. *Other Structures*

Stigma. In the light microscope image of living flagellates, the stigma appears frequently as one body intensely colored orange-red. In the electron microscope, however, it shows as an agglomeration of numerous, dense, and randomly distributed granules which vary in size from 100 to 300 mμ and which appear homogeneous at the present level of resolution. In *Euglena*, as shown in PLATE 2, FIGURES 9 and 10, the granules of the stigma are located immediately below the membrane of the reservoir, a chamber with smooth walls that follows the ridged gullet and in which the initial part of the flagella are found. Between the granules and the membrane of the reservoir one encounters a system of more or less regularly spaced fibrillae (PLATE 2, FIGURE 10) which seem to be the expansion of the fibrillae disposed longitudinally around the deepest part of the gullet and shown in PLATE 3, FIGURE 12. Occasionally, one finds a dense, homogeneous body attached to one of the flagella and facing the stigma. On account of these features it can be identified as the paraflagellar body or the photoreceptor. As known, the stigma contains besides carotenoids, a special pigment, namely astaxanthin (a dehydroxydiketo derivative of β-carotene), which has been encountered thus far only in animals. In this respect, *Euglena* occupies a very interesting position in that it is the only organism known to possess both plant and animal pigments.[22]

Flagella. There are two flagella for each of the protists studied, but the behavior of the second one is highly debated in *Euglena* and was not rendered clearer by our study. In sections, the flagella appear to consist of a number of elementary filaments (axonemata) embedded in a matrix and covered by a common membrane (PLATE 2, FIGURE 10). The appearance of the basal body in *Poteriochromonas* can be seen in PLATE 3, FIGURE 13. In favorable sections, the number of elementary filaments can be counted and usually appears to be 11 in both flagellates. Nine of these filaments are peripherally located, while the other two are found as a pair in the center of the flagellum. Sometimes the central filaments are missing. In the organization of the flagella one again encounters a general pattern that seems to prevail for all cilia and flagella of animal cells[23] from protists to mammals. The existence of elementary filaments in the flagella of *Euglena* has already been reported in an electron microscope study of whole, mounted specimens.[24] In addition, lash-like expansions mastigonemata) have been seen around these flagella,[24] and the fine, divergent filaments that appear in PLATE 2, FIGURE 10 around the main flagellum may well represent such appendages.

Pellicle. The membrane of *Euglena* appears to have differentiated an exoskeleton consisting of a system of semi-rigid rings (PLATE 3, FIGURE 11) that alternate with strips of soft, pliable membrane (PLATE 2, FIGURE 9). It is difficult to ascertain from the electron micrographs if the arrangement of the rings is circular or spiral. The examination of a large number of pictures of *Euglenas* in various states of contraction gives the impression that the rings could slide into one another in the same manner as do, on a much larger scale, the abdominal rings of insects.

Summary

Direct evidence for the existence of a laminated structure in chloroplasts has been found in two flagellates, namely, *Euglena gracilis* and *Poteriochromonas stipitata*. Other cell organelles, such as mitochondria, endoplasmic reticulum, and flagella were found to be organized according to the general patterns that appear to prevail in all animal cells. The morphology of these protozoa, especially of *Euglena*, and the fate of their chlorophyll during dark- and light-adaptation has been studied and, while the observations on the adaptation process are still far from complete, the data available suggest that chlorophyll is not entirely lost in cells reversibly adapted to darkness and that chloroplast remnants may still persist in flagellates re-adaptable to light.

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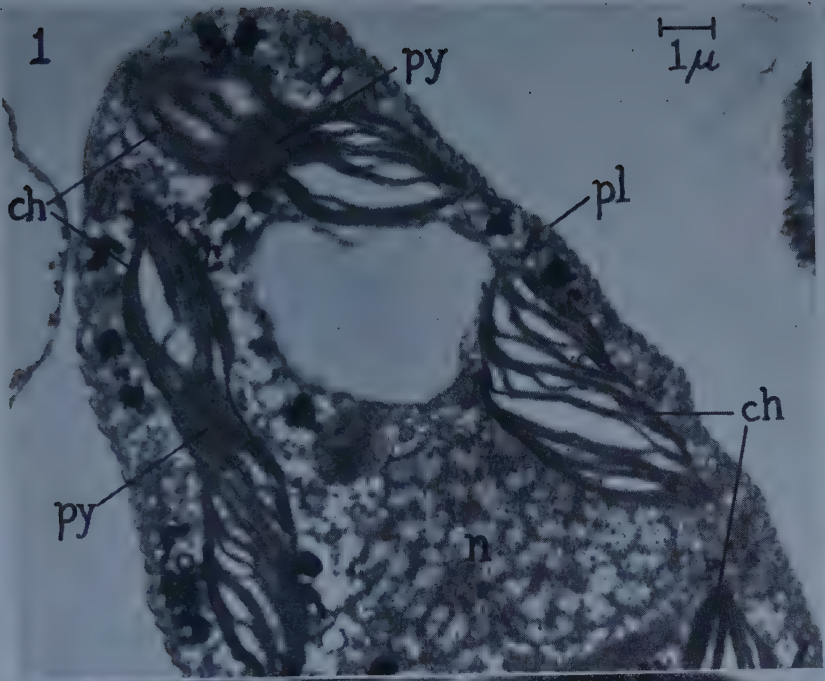
PLATE 1

FIGURE 1. A large area of a longitudinal section through an active form of *Euglena* is shown in this figure, in which the ridged pellicle (*pl*), the nucleus (*n*), and a number of chloroplasts (*ch*) can be recognized. The pyrenoid (*py*) can be seen in two of the chloroplasts. Magnification: 7500 X. Fixation in 1 per cent OsO_4 at pH 6.2.

FIGURE 2. Shows part of the profile of a chloroplast with most of the composing lamellae (*l*) cut perpendicularly. A few granules (*g*) appear in one of the interlamellar spaces. Magnification: 30,000 X.

FIGURE 3. Illustrates a chloroplast of an inactive form of *Euglena*. Note the irregular disposition of the lamellae (*l*) and the large and numerous chloroplast granules (*g*). Magnification: 9000 X.

FIGURE 4. Shows a chloroplast of an active form of *Euglena* with the lamellae sectioned very obliquely. This provides a clearer image of the limiting membrane of the organelle. Magnification: 10,500 X.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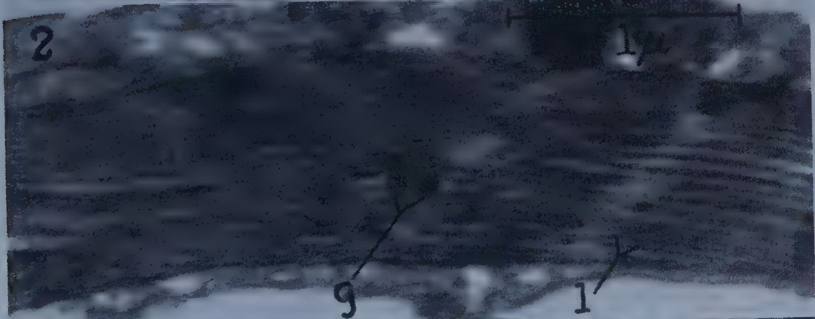
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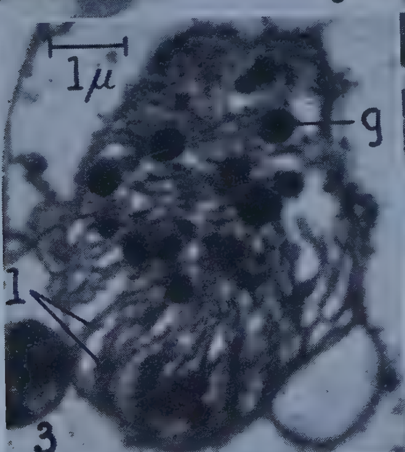
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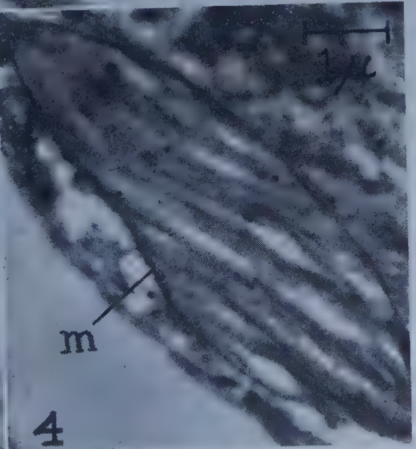
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FIGURE 5. Shows a series of absorption curves obtained during the adaptation of a culture of colorless *Euglenas* to light. Aliquots were taken at the time intervals indicated for each curve and extracted with 80 per cent acetone. The absorption curve of the extracts were taken in a Carey recording spectrophotometer.

FIGURE 6. Illustrates a reverse experiment; namely, one in which a culture of green *Euglenas* was adapted to darkness, timing, extraction and spectrophotometry as in FIGURE 5.

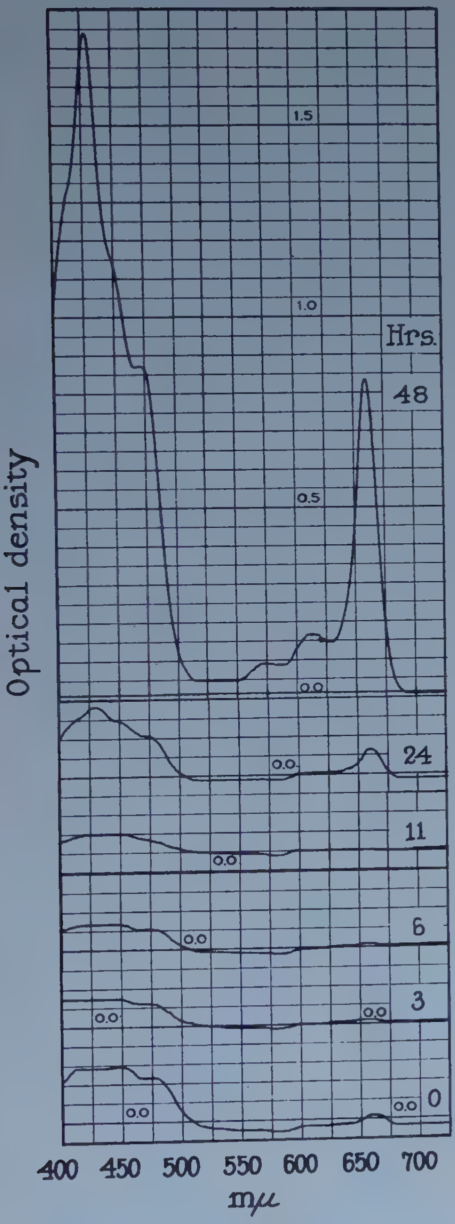


FIGURE 5

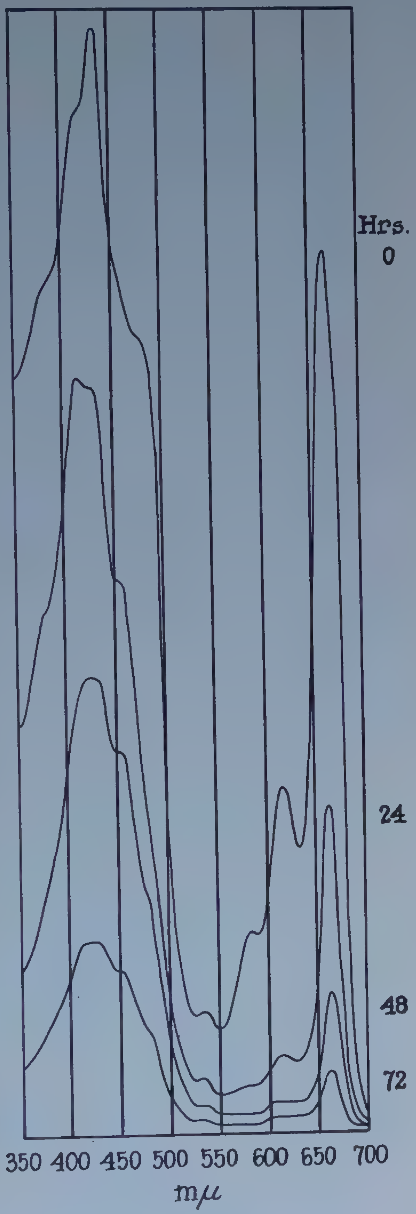


FIGURE 6

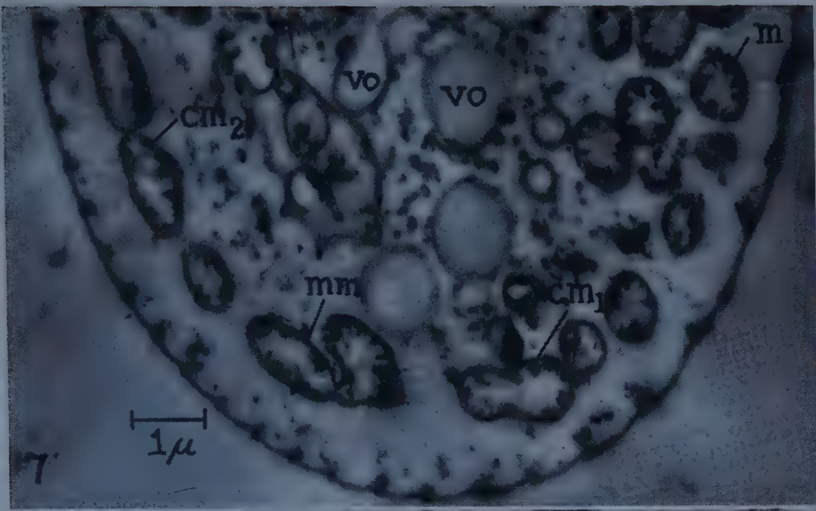
PLATE 2

FIGURE 7. Illustrates part of a section through a dark-adapted *Euglena*. Numerous mitochondria (*m*), most of them marginated, appear in the profile of the protist. They show their limiting membrane (*m.m*) and their cristae mitochondriales (*c.m*), which, because of a certain amount of irregularity in their orientation, can be seen either from the edge ($c.m_1$) or from the side ($c.m_2$). A number of vacuoles (*vo*) are visible in the central part of the section. The apparent variation in electron density and "thickness" exhibited by their membranes is referable to the degree of obliqueness at which the plane of the section cuts through the membrane. The same applies for the mitochondrial membranes. Magnification: 9000 X.

FIGURE 8. Shows a limited area in the cytoplasm of a dark-adapted *Euglena* with a few mitochondrial profiles (*m*) and some elements of the endoplasmic reticulum. The latter appear as a bundle of tubular elements (*t*) or as a scattering of apparently isolated vesicles (*v*). Magnification: 25,000 X.

FIGURE 9. Represents the stigma (*s*) of an active *Euglena* grown in the light. Note the position of the component granules between the pellicle (*pl*) and the reservoir (*r*), in which the profiles of the two flagella (*f*) are visible. At *pl* the pellicle shows well the alternation of apparently rigid and pliable strips. Magnification: 16,500 X.

FIGURE 10. This is an oblique section through the stigma showing its component granules (*sg*), the fibrillar system of the reservoir (*fb*), and the flagella. In one of the latter the axonemata (*ax*) can be seen. The filaments marked *mg* may represent the mastigonemata of the flagella. Magnification: 16,500 X.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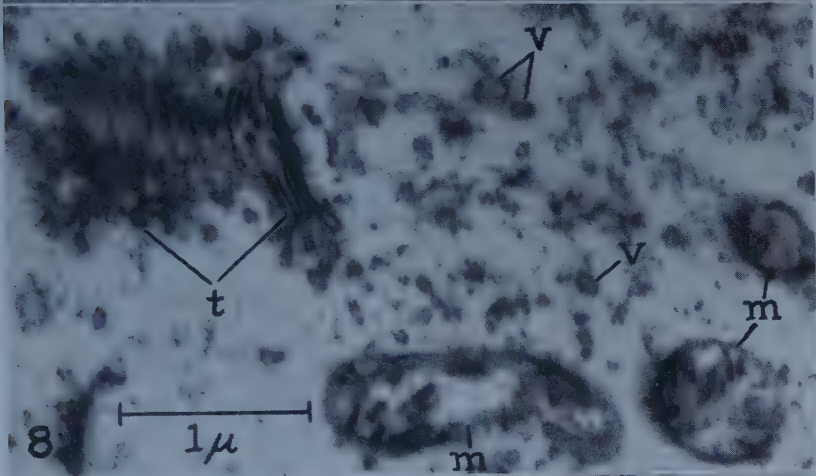
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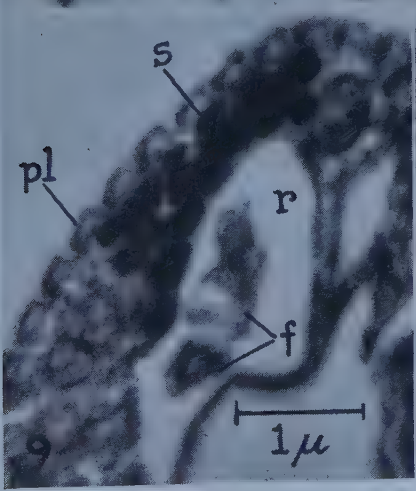
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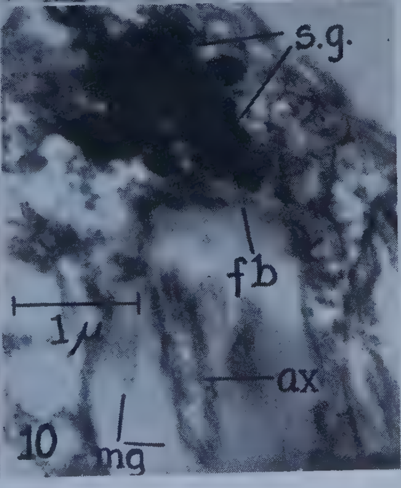
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Plate 3

Figure 11. This is a longitudinal section through the body of an active *Euglena*. The protist was slightly bent and the section passed through the concave side very close to its surface. The rigid rings (*r*) of the pellicle show clearly their truncated, conical shape. In the middle region of the profile, the pliable strips of membrane that connect the rings have not been touched by the section, so that the rings appear isolated from one another. Magnification: 10,000 X.

Figure 12. Shows the anterior pole of a *Euglena* where the ridges of the pellicle become finer (*a.p*) close to the entrance in the gullet. The entrance itself does not appear in the picture but an oblique section through the gullet can be seen at *g*. A system of longitudinal fibrillae (*fb*) can be noticed in the wall of the gullet. Around the latter appear two successive coronas, one (c_1) apparently made up of a network of very fine fibrils and the other (c_2) of elements of the endoplasmic reticulum. A few mitochondria showing their membranes and cristae can also be seen in the picture. Magnification: 14,000 X.

Figure 13. Show parts of the profiles of two chrysomonads (*Poteriochromonas stipitata*). The one to the left shows the nucleus (*n*), a large chloroplast (*ch*), and a flagellum (*f*) with its component axonemata and basal body (*bb*). Note the change in appearance of the chloroplast from its upper right to its lower left end. It describes a change in the orientation of the lamellae in relation to the plane of the section. They are cut perpendicularly in the upper right half and very obliquely in the lower left one. The flagellate to the right shows the nucleus with the two adjacent chloroplasts, a few mitochondria (*m*), and, immediately below the membrane, a vacuolar cavity (*vo*), which may be a contractile vacuole. Magnification: 17,500 X.

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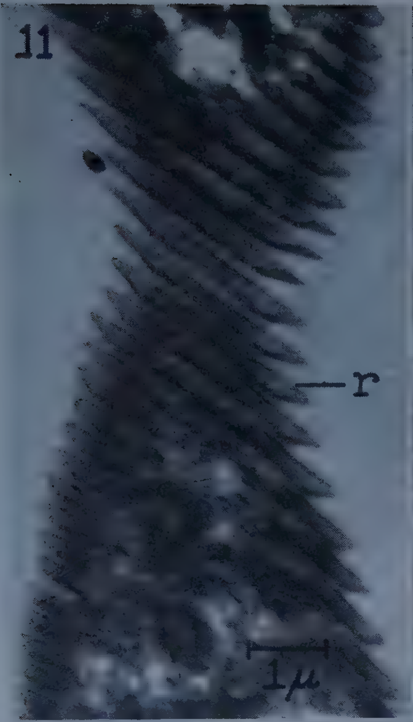

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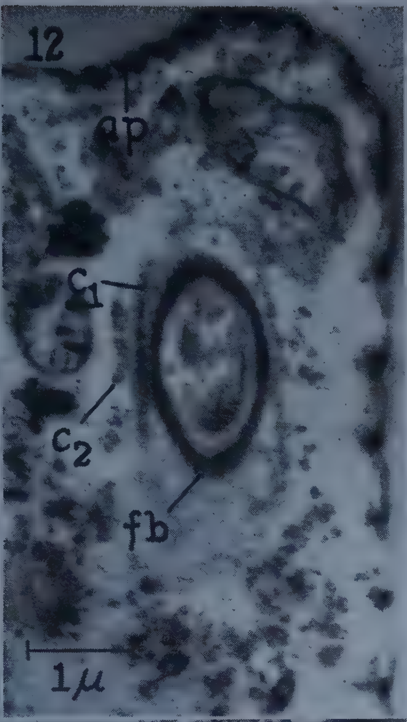

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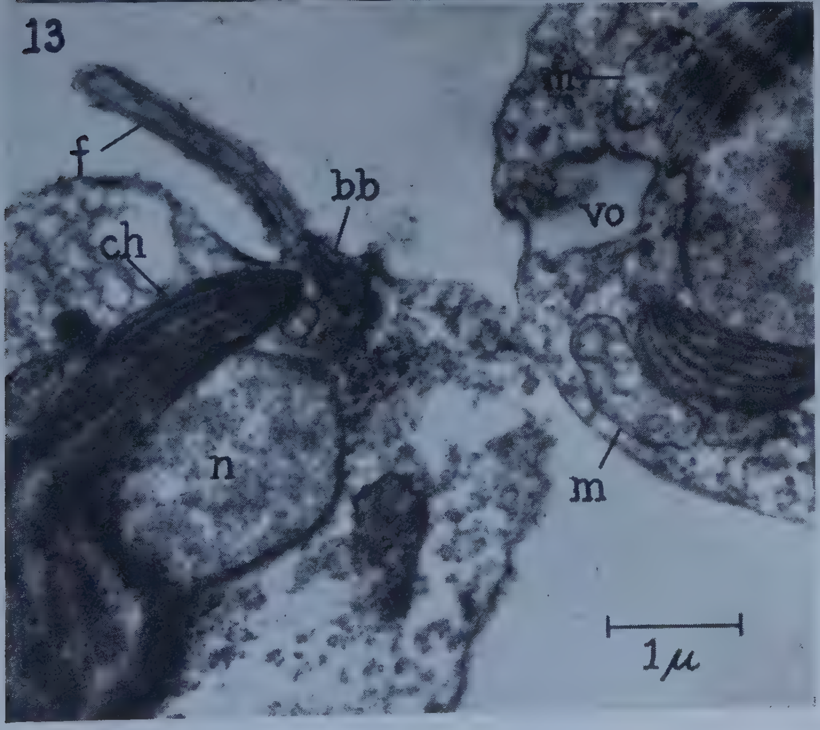

CO_2 FIXATION BY *EUGLENA*[*]

By V. H. Lynch and M. Calvin

Radiation Laboratory and Department of Chemistry, University of California, Berkeley, California

During a comparative study of the dark CO_2 fixation by microorganisms, one organism was found to differ greatly from all others investigated.[1] This microorganism was the green flagellate, *Euglena gracilis*.

Euglena has long been a controversial organism. It is claimed both by the botanists and the zoologists since it has many of the characteristics of both a plant and an animal and is often placed in the phylogenetic tree on the fork at which the plant and animal kingdoms are conceived to have separated. The biochemist has been interested in its metabolism because of its ready utilization of acetate and its apparent inability to utilize sugars.[†] Although *Euglena gracilis* does not use sugars, Albaum *et al.*[2] have shown that the phosphorylated compounds of the glycolytic cycle are present in *Euglena*.

In order to extend our knowledge of some of the metabolic reactions of *Euglena*, a brief survey using radioactive-tracer techniques was undertaken.

Methods

The organism used in this investigation was *Euglena gracilis* var. *bacillaris* obtained from Doctor S. H. Hutner of the Haskins Laboratories. The following growth medium was used: 5 g. Difco tryptone, 5 g. sodium acetate$\cdot$3 H_2O, 0.5 g. NH_4NO_3, 0.5 g. $MgSO_4\cdot 7\ H_2O$, 0.5 g. K_2HPO_4, 1 ml. of an iron solution containing 0.5 per cent $FeSO_4\cdot 7\ H_2O$ and 0.2 per cent tartaric acid, and 1 liter distilled water. The pH was adjusted to 7.0 with NaOH. After inoculation, the culture was placed under constant illumination and shaken occasionally. The cells were harvested after growth periods of three to seven days.[‡]

Cell suspensions for use in the radioactive carbon experiments were prepared in the following manner. The cells were removed from the growth medium by centrifugation and washed once with distilled water. A 1 per cent cell suspension was prepared by suspending 1 ml. of these packed cells in 100 ml. of distilled water.

Dark CO_2 Fixation. Fifty ml. of a 1 per cent cell suspension were placed in 125 ml. glass-stoppered Erlenmeyer flasks. The flasks were wrapped with aluminum foil and placed in a dark room. All subsequent operations were performed using a yellow safety light. Radioactive carbon was added to the cell suspensions as a sodium bicarbonate solution containing 0.0248 ml. M $NaHCO_3$ and 200 μc. C^{14} per ml. 0.1 ml. of this bicarbonate solution was the amount usually added per experiment. The flasks were then stoppered, shaken occasionally during the exposure period and the cells were killed by dumping into a volume of boiling absolute alcohol to give a final concentration of 80 per cent ethanol.

Photosynthetic Experiments. One per cent cell suspensions were placed in a lollipop[3] and exposed to a light intensity of 7,400 lux. Air was bubbled through for 15 min. before addition of the radioactive bicarbonate solution. The lollipop was stoppered, and the suspension was constantly shaken in the light beam for a measured time period and then rapidly poured into boiling ethanol to give a final concentration of 80 per cent ethanol.

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[*] The work described in this paper was sponsored by the U. S. Atomic Energy Commission.

[†] Recent work by Cramer and Myers has indicated that *Euglena gracilis* can be trained to utilize glucose.

[‡] The authors wish to thank Mrs. Louisa Norris for her assistance in growing these organisms.

Radioactivity Measurements. All radioactive experiments were analyzed in the following manner. Immediately after killing in boiling ethanol, the suspensions were cooled, the volume was determined, an aliquot of the entire suspension plated on aluminum plates and the activity counted using a Geiger-Müller counting tube. In this manner, the total amount of radioactivity incorporated into nonvolatile, stable compounds was determined. The cell extracts were acid enough so that all unchanged carbon dioxide was removed during the plating procedure. The cell suspension was then filtered through Celite and the activity of the filtrate determined in the same manner as for total activity. This value is reported as the soluble activity or the 80 per cent alcohol-soluble activity.

Location and Identification of Products. Paper chromatography of the 80 per cent alcohol extract was employed to determine the individual compounds which contained radioactivity. The alcohol extract was concentrated *in vacuo* at $<40°$ to a small volume ($\sim$2 ml.) and then aliquots placed on Whatman paper No. 1.

Two-dimensional chromatograms using water-saturated phenol as the first solvent and butanol (37.5 ml.)–propionic acid (17.6 ml.)–water (24.9 ml.) as the second solvent were run and the radioactive areas of the paper located by use of x-ray films. Each radioactive area was identified either by cochromatography with a known compound or by chemical treatment of the areas after elution from the paper and identification of the resulting products. The amount of radioactivity in each spot was determined by direct counting on the original chromatogram with a thin-window Geiger-Müller counting tube.[1]

Experimental Procedure and Results

Dark CO_2 Fixation. The radioactive products formed during dark CO_2 fixation by *Euglena gracilis* were strikingly different from those of any other microorganism, either photosynthetic or nonphotosynthetic, that has been investigated[1] (FIGURE 1). Not only were the characteristic dark CO_2 fixation products (malic, citric, aspartic, and glutamic acids) formed, but many of the phosphorylated compounds typical of CO_2 fixation in the light were observed. See TABLE 1.

The normal procedure for a dark CO_2 fixation experiment is to add the radioactive bicarbonate solution to the cell suspension immediately after placing the cells in darkness. When *Chlorella* or *Scenedesmus* in the absence of CO_2 is exposed to high light intensities and then immediately placed in the dark and CO_2 added, it is capable of fixing an appreciable amount of CO_2 into phosphorylated compounds, the "pre-illumination effect."[4] The compounds formed are qualitatively the same as those produced by photosynthesis. To test whether the dark fixation of CO_2 into phosphorylated compounds by *Euglena* might also be a "preillumination effect," the following experiment was performed:

The washed cells were resuspended in distilled water and air was bubbled through the suspension while it was kept in the dark for 16 hours. After this time, radioactive CO_2 was added while the suspension was still in the dark and

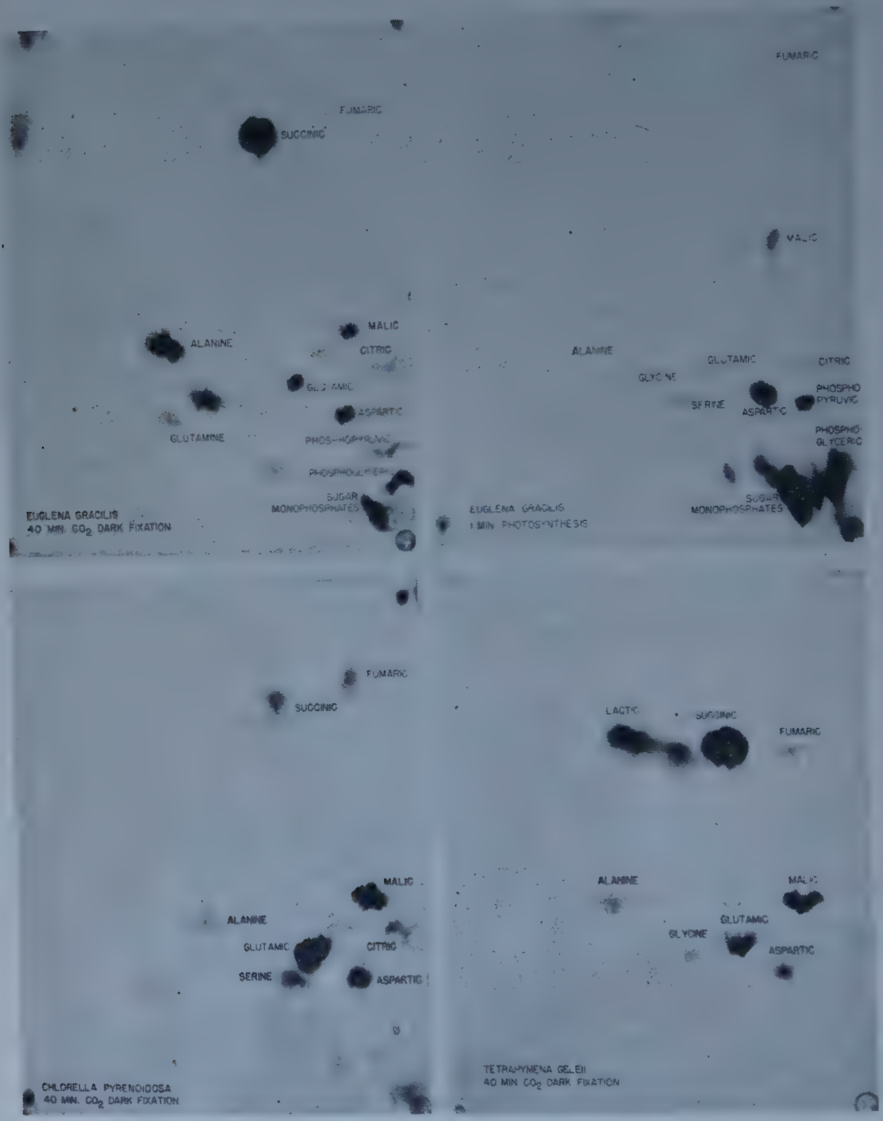


FIGURE 1. Patterns of light and dark CO_2 fixation by *Euglena* compared to dark fixation by two other organisms.

the cells were killed after 40 minutes. All operations were carried out in total darkness. Chromatograms from this experiment again showed that phosphorylated compounds as well as the usual organic acids and amino acids, had become radioactive (TABLE 1, column 2). Therefore, the dark fixation of CO_2 into phosphorylated compounds by *Euglena* does not involve the use of energy stored in the cells from a previous exposure to light as in "preillumination."

The radioactive phosphates were identified by chromatographic position of the phosphate and of the carbon compound obtained after enzymatic hydrolysis with Polidase-S. The same radioactive phosphates were found to be present after dark CO_2 fixation as are formed during photosynthesis. Even sedoheptulose phosphate, identified by conversion of sedoheptulose into sedoheptulosan by acid treatment, was found to be radioactive.

Phosphoglyceric acid accounted for five per cent of the total soluble activity and the sugar monophosphates for 25 per cent of the soluble activity. The organic acids and amino acids represented 43 per cent of the fixed soluble activity. Serine and glycine, formed only in trace amounts in most dark fixation experiments with other organisms, accounted for 12 per cent of the activity.

TABLE 1
RADIOACTIVE PRODUCTS FORMED DURING A 40 MIN. DARK CO_2 FIXATION BY *Euglena gracilis*

Product	Per cent of 80 per cent alcohol soluble* activity	
	No dark adaptation	16 hrs. dark adaptation in air
Fumaric	1.2	0.8
Succinic	21.3	41.2
Malic	4.8	3.4
Citric	5.0	2.7
Glutamic	6.5	3.4
Aspartic	2.7	4.6
Alanine	2.7	10.5
Phosphopyruvic	1.1	0.3
Serine and glycine	12.4	9.6
Phosphoglyceric	4.6	6.0
Sugar monophosphates	19.8	16.0
Nucleotides	5.4	—

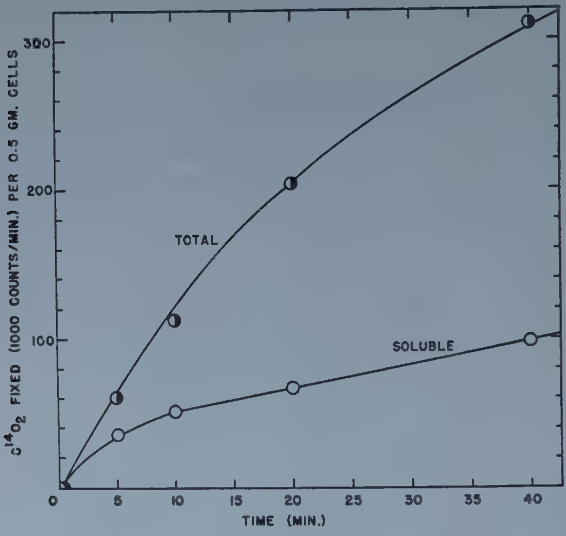
* Per cent of total fixed activity soluble in 80 per cent alcohol approximately 50 per cent.

Several experiments were conducted in an effort to determine whether CO_2 might be entering the metabolic pathways of *Euglena via* two distinct routes or whether carbon dioxide appearing in sugars had first been fixed into organic compounds via reversal of the tricarboxylic acid cycle.

The rate at which CO_2 was fixed in the dark was determined over the time interval of 5 to 40 minutes. The amount of activity fixed during this time period is shown in FIGURE 2. Both the total amount of activity fixed and the activity fixed in 80 per cent alcohol-soluble compounds are plotted. The total amount of activity increases steadily with time although at a decreasing rate. The alcohol soluble compounds increase rapidly for the first 10 minutes with a very slow increase at later time. The two different fixation rates, particularly in the soluble components, suggests two simultaneous different routes of CO_2 incorporation.

Paper chromatography was used to separate and identify the compounds present in the alcohol-soluble fraction. The amount of radioactivity in each compound was also determined by direct counting of the compounds on the paper chromatograms. Thus, the amount of activity in each compound as a function of time could be determined. The results are plotted in FIGURE 3.

The sugar monophosphates, giving primarily glucose, fructose, and sedoheptulose on hydrolysis, contain the largest amount of radioactivity at the shortest time tested. Their activity continues to remain the major fraction at all of the longer time periods investigated. A nucleotide giving glucose as the principle radioactive compound on phosphatase treatment also contains a large fraction of the activity. This has been shown to consist largely of uridine diphosphate glucose.[5, 6] Phosphoglyceric acid contains considerable activity but less than the sugar phosphates even at the five-minute interval. Of the non-phosphorylated compounds, the combined activity of glutamic acid and glutamine has the highest value. Aspartic and malic acids have very low activities.



EUGLENA – DARK CO_2 FIXATION

FIGURE 2. Time course of dark CO_2 fixation by *Euglena*.

Another experiment designed to elucidate the manner in which CO_2 is incorporated into metabolic pathways in the dark involved treatment of the cells with malonate to inhibit succinic dehydrogenase. *Euglena* cells were suspended in 0.05 *M* malonate at pH 4.3 for 105 minutes and then radioactive carbon dioxide added for 15 minutes. Under these conditions, malonate alters the fixation pattern of *Scenedesmus*.[7] After this treatment the cells were filtered rapidly through celite and killed by pouring boiling 80 per cent ethanol through the algae. A control experiment, identical except for the omission of malonate was also carried out. The amount of alcohol-soluble radioactivity fixed was the same in both samples. Determination of the activity of individual compounds also shows that the malonate treatment had no significant effect on the distribution of activity within the individual compounds (TABLE 2).

When radioactive carbon dioxide is administered to photosynthetic or non-photosynthetic tissues, radioactive sugars may be found. The distribution of activity in the individual carbons of the sugar skeleton, however, is strikingly different for the two types of reactions. The radioactive glucose obtained by hydrolysis of the phosphates formed by *Euglena* during a 40-minute carbon dioxide fixation in the dark was oxidized with periodate.* Only the carbon of the primary alcohol group of glucose will be oxidized to formaldehyde by this treatment. This formaldehyde was trapped with dimedon and its activity determined. The formaldimedon contained very little radioactivity (less than 0.1 per cent). The activity of the other carbon atoms was not determined. If carbon dioxide were fixed in glucose by the same reactions as occur during photosynthesis, the number 6 carbon of glucose so produced would be radioactive. In order to estimate just how much radioactivity one might expect to find in this carbon atom, it is necessary to determine approximately what fraction of the labeled glucose found in a 40-minute dark fixation might have

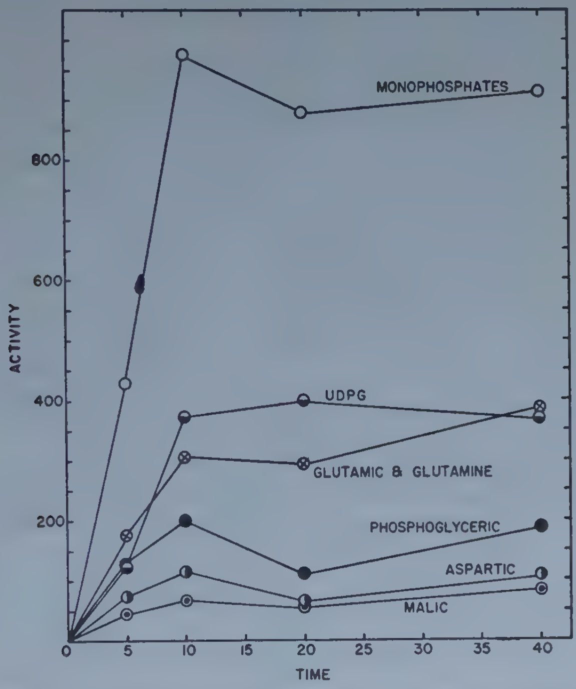


FIGURE 3. Time course of dark CO_2 fixation in various compounds by *Euglena*.

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* We wish to thank Doctor J. A. Bassham and Mrs. A. Harris for performing these oxidations.

been formed by a route corresponding to that occurring during photosynthesis. This may be estimated as that corresponding to the rapid rise in the soluble curve of FIGURE 2, which would be about 8×10^4 c.p.m./g. cells. This is an amount of soluble activity that would correspond to about 20 seconds of photosynthesis as may be seen from TABLE 3.

The shortest photosynthesis experiments available for *Euglena* was 1 minute and degradation of some glucose from these gave C_6 ~13 per cent and $C_4 + C_5$ ~40 per cent. This is approximately the same distribution as was obtained in a similar experiment with *Scenedesmus*, so that presumably the rate of photosynthesis labeling in the two organisms are comparable. Hexose from 30 sec. photosynthetic experiments with *Scenedesmus* shows C_6 ~3 per cent. If this figure is extrapolated back to ~20 sec. of photosynthesis and then diluted by an equal amount of glucose formed by reversible terminal carboxylation reactions and labeled only in C_3 and C_4, it is clear that the existing data do not eliminate the possibility of an appreciable fraction of the glucose found in 40 min. dark fixation by *Euglena* having been formed by a mechanism similar to that used in the light reaction. Of course, the possibility of still another mode of CO_2 fixation being available in *Euglena* is very likely.

TABLE 2

EFFECT OF MALONATE ON 15 MIN. DARK CO_2 FIXATION BY *Euglena*

	Control, per cent of soluble activity	Malonate treated, per cent of soluble activity
Succinic	9.6	8.2
Fumaric	1.5	—
Malic	3.4	4.6
Citric	2.7	4.0
Glutamic	10.6	11.5
Glutamine	3.4	3.2
Alanine	10.3	9.3
Aspartic	6.4	6.9
Phosphates	43.6	43.4

TABLE 3

AMOUNT OF $C^{14}O_2$ FIXED BY ONE GRAM OF *Euglena* CELLS

	1 min. PS c/m/g. cells	5 min. PS c/m/g.	5 min. dark c/m/g.	10 min. dark c/m/g.	20 min. dark c/m/g.	40 min. dark c/m/g.
Total activity	387,000	1,960,000	118,000	224,000	406,000	620,000
Soluble activity 80% alcohol	290,000	877,000	73,000	102,000	132,000	196,000

Photosynthesis Experiments. Photosynthetic experiments of one and five minutes exposure to radioactive CO_2 were performed. The majority of activity, 84 per cent in one minute, 70 per cent in 5 minutes, is found in phosphorylated compounds (TABLE 4). The general photosynthetic pattern for *Euglena* is similar to that of other photosynthetic organisms.[8]

Euglena gracilis is known as one of the acetate flagellates, preferring acids and alcohols to sugars as a carbon substrate. The organisms used in our laboratory are grown in the presence of both light and acetate. Therefore, it was of interest for several reasons to investigate the utilization of acetate by resting cells in the presence or absence of light. One mg. of sodium acetate-2-C^{14} was added to a suspension of 500 mg. wet weight of *Euglena* cells in 100 ml. of distilled water. Four per cent CO_2 in air was continuously bubbled through the suspension to remove any carbon dioxide that might be formed by decomposition of the acetate. Determinations of the total activity at the end of both the light and dark experiments (after 12 minutes) showed that none of the radioactivity had been lost during the experiment. Therefore, the methyl carbon of the acetic acid was not oxidized to carbon dioxide, or, if converted to CO_2, it was reutilized by the cells before liberation into the external medium for the cells.

Table 4

$C^{14}O_2$ Fixation During 1 min. and 5 min. Photosynthesis by *Euglena gracilis*

	Per cent soluble activity			Per cent soluble activity	
	1 min.	5 min.		1 min.	5 min.
Succinic	—	0.4	Nucleotide phosphate	12.5	19.6
Fumaric	0.4	2.2	Dextrins	1.1	4.1
Malic	1.2	—	Glutamic	0.1	1.5
Citric	0.5	0.3	Serine	0.6	4.1
Aspartic	9.3	8.5	Glycine	0.4	5.4
Phosphopyruvic	0.9	0.8	Alanine	0.5	0.6
Phosphoglyceric	16.0	7.6	Per cent insolubles	25	55
Sugar monophosphates	54.7	42.5			

Table 5

$C^{14}H_3COOH$ Utilization (in 12 Minutes) by *Euglena*

	Activity fed, cts./m	Activity fixed, cts./m	
		Total	80 Per cent alcohol soluble
Light	8,200,000	1,100,000	230,000
Dark	8,200,000	400,000	250,000

The total amount of acetate radioactivity converted to stable, nonvolatile compounds was determined by plating and flooding the plate with glacial acetic acid. This last step was necessary to insure complete removal of unchanged acetic acid. The 80 per cent alcohol-soluble activity was determined in an identical manner. The results are shown in TABLE 5.

These results show that a considerable amount (13.4 per cent) of the acetate fed was converted to other organic compounds during a twelve-minute period of photosynthesis. In the light, there is a greater utilization of acetate than in the dark. Although more acetate was utilized in the light, the same amount of activity was found in the 80 per cent alcohol-soluble fractions of the light and dark experiments. Chromatograms of the alcohol-soluble materials indicate that, in the light, the greatest fraction (47 per cent) of this activity was in the lipid and phospholipid fraction. In the dark, only 18 per cent of the alcohol-extractable incorporated radioactivity is found in the fat fraction, with 50 per cent of the fixed activity present as phosphorylated compounds (TABLE 6). This enhanced incorporation of acetate into lipid under the influence of light is similar to that found earlier for green algae[4] (*Scenedesmus*) and purple bacteria,[9]* (*Rhodospirillum rubrum*).

TABLE 6

ACETATE-2-C^{14} FEEDING EXPERIMENT

	Per cent of 80 per cent alcohol soluble activity			Per cent of 80 per cent alcohol soluble activity	
	Light*	Dark*		Light*	Dark*
Aspartic	8.1	5.2	Phosphates	20.7	49.3
Glutamic	4.9	7.8	Dextrins	7.3	3.7
Glutamine	3.1	5.8	Lipids and phospholipids	47.4	17.3
Alanine	4.8	5.5			

* 12 min. exposure to acetate-2-C^{14}.

Discussion

That characteristic of the dark fixation of carbon dioxide by *Euglena*, which is different from that in all other organisms so far examined, is most clearly represented in FIGURE 1. It is clear from this that the pattern of compounds into which carbon dioxide is fixed in the dark by *Euglena* is very similar to that of photosynthetic carbon dioxide fixation. This is not true of any of the other tissues so far examined in which the animal kingdom is represented by liver slices and a colorless protozoon, and the plant kingdom represented by yeast and water molds, a variety of aerobic and anaerobic bacteria, green algae, and leaves from higher plants. Although the data available to us at present regarding the distribution of the labeled carbon in compounds produced by the dark fixation do not permit us to say unequivocally that they have been formed by routes identical with or closely parallel to those used in photosynthetic fixation, there is some indication that this may be so. In addition to the degradation data on glucose already discussed, there is one other bit of information on the malic acid formed during 10 minute and 40 minute dark fixations by *Euglena*.† The 10 minute malic acid shows approximately 18 per cent of its radioactivity in the α-carboxyl carbon with all of the remaining activity (approximately 72 per cent) found in the β-carboxyl carbon. On the other hand, the 40 minute malic acid shows 25 per cent in the α-carboxyl and about 65 per cent in the β-carboxyl, leaving approximately 15–25 per cent in the two central carbon atoms of malic acid. This distribution would indicate that a small amount of this malic acid had been formed by a reaction similar to those by which it is formed in the light. In contrast to this, radioactive malic acid obtained from *Chlorella* which have fixed radioactive carbon for 40 minutes in the dark shows approximately 50 per cent of the radioactivity in the α-carboxyl carbon and the remaining 50 per cent in the β-carboxyl carbon.

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* Unpublished observations in this laboratory.

† These degradations were performed by Doctor P. K. Christensen, Fellow of the Norwegian Council on Scientific Research, to whom we here express our thanks.

Even if it is not possible for us to say precisely by what mechanism the carbon dioxide finds its way into the photosynthetic intermediates in the dark, it is quite clear that the coupling of energy, made available by respiration and/or fermentation, with carbon dioxide reduction, exists in *Euglena* to an extent not found in any other tissue examined, either plant or animal. A further evidence of this close connection between dark and photosynthetic metabolic routes in *Euglena* may be seen in the behavior of labeled acetate. In *Euglena*, labeled acetate in the dark finds its way not only into tricarboxylic acid intermediates but into all of the compounds normally found as intermediates of photosynthetic CO_2 assimilation. This is in contrast to the situation in *Scenedesmus* in which acetate carbon in the dark finds its way only into the tricarboxylic acid intermediates.

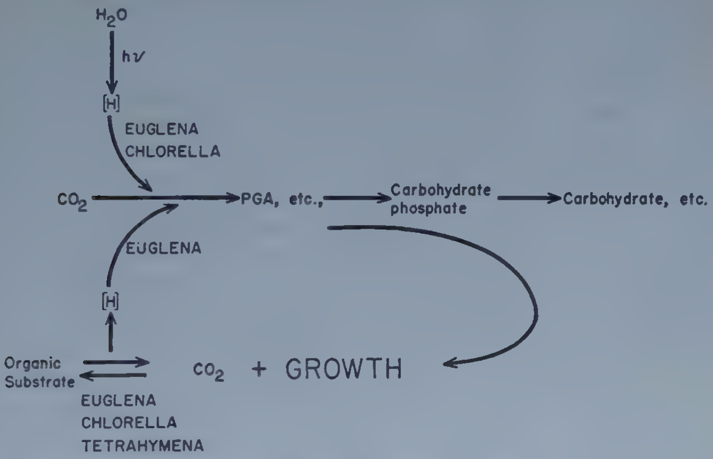

FIGURE 4. Diagrammatic representation of possible enzymatic interrelationships among several organisms.

Assuming for the moment that the mechanism for carbon dioxide incorporation in the dark includes metabolic pathways very similar to those used in the light, one can represent the relationships between the *Euglena* studied in this work with green plants as represented by *Chlorella* and animal organisms represented by *Tetrahymena* as shown on the chart (FIGURE 4). Here it is clear that the ability to utilize chemical energy for carbon dioxide fixation in a manner similar to that by which photochemical energy is used is limited to *Euglena*. It is capable of carrying out all three types of metabolic sequence in contrast to the other two types of organisms represented by *Tetrahymena or Chlorella*.

It is interesting to speculate upon the possible significance of these facts with respect to the phylogenetic relationships of *Euglena*.[10] They could be used to confirm the old notion based upon morphology and gross physiology that *Euglena* represents the type of parent at which the separation of the plant and animal kingdom took place. We could suggest that the evolution toward the animal kingdom involved the loss of not only the photochemical apparatus but the ability to reduce carbon dioxide by the path of carbon dioxide fixation. This does not imply that all the animal kingdom as we know it today must have passed through photosynthetic organisms in their evolution. It is quite conceivable and altogether likely that many of them have evolved directly from the primitive heterotrophic, dependent reproductive units. The distinction between those which have and those which have not passed through a photosynthetic stage in their evolution may eventually be found on the biochemical level in terms of carbon dioxide fixation patterns or some similar criterion.

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NUTRITION OF *PERANEMA**

By Jack Storm and S. H. Hutner
Haskins Laboratories, New York, N. Y.

Animals, designated broadly as phagotrophs, are diverse in origin. Organization of their comparative biochemistry is well under way based largely on data from a few metazoa (mammals, birds, and insects) and ciliates (*Tetrahymena* and *Paramecium*). Theories about the biochemical features characterizing animals, if derived exclusively from species remote from their presumably photosynthetic ancestors, are too narrowly founded. Later specializations reflecting a restricted range of prey, or adaptations to cellularity may obscure common features. Certain flagellate groups comprise both phototrophs and phagotrophs and are therefore of special value. Indeed, in certain chrysomonads, phagotrophy and phototrophy co-exist in the same organism. But before generalizing about which components of the photosynthetic apparatus survive in the dark metabolism of phagotrophs, other plant→animal groups, such as the Euglenineae, should be looked into. In recent years, understanding of the nutrition and intermediary metabolism of phototrophic euglenids has deepened, but the animal euglenids remained untouched. Consequently, when Professor E. G. Pringsheim presented us with a pure culture of the colorless voracious euglenid *Peranema trichophorum*, it provided the opportunity, for which we are most grateful, to examine the nutrition of a phagotroph belonging to a line rich in (1) photosynthetic species widely divergent in capacity for heterotrophic nutrition; (2) colorless osmotrophs; and (3) phagotrophs. No photosynthetic euglenid is known to be phagotrophic; in this respect, the euglenid series is incomplete as compared with the chrysomonads and dinoflagellates.

The use of *Euglena gracilis* for assaying cobalamin lent a practical incentive to this investigation. *Euglena* is limited in ability to utilize high-molecular cobalamin—a shortcoming of the assay.[7, 8, 9] If a cobalamin requirement is phyletic in euglenids, then *Peranema* should need cobalamin and, as a phagotroph, serve for assaying total cobalamin in high-molecular materials, even in intact cells, without chemical or enzymatic digestion being needed to render the vitamin assayable.

Chen[1] first cultivated *P. trichophorum* on living *Saccharomyces exiguus* + soil extract. Thriving cultures were then obtained on autoclaved cows' milk. Chen's observations depict it as strikingly omnivorous and aggressive—in his words:

> "*Peranema* is capable of ingesting a great variety of living organisms providing these are motionless. Small organisms are swallowed whole; larger ones are either engulfed or cut open by the rod-organ and their contents sucked out. The rod-organ can be protruded out of the cytostome and used in holding on to, and cutting, the periplast of the prey. Starch-grains, oil-droplets, and protein-particles are engulfed and digested."

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* This investigation was aided by grants from the Lederle Laboratories Division of the American Cyanamid Company, the Atomic Energy Commission, Contract AT(30-1)-863, and the Rockefeller Foundation.

The present paper describes how some requirements of Chen's strain were identified.

Methods

P. trichophorum is obtainable in England from the Culture Collection of Algae and Protozoa, the Botany School, Downing St., Cambridge.

As noted by Chen, *Peranema* moves in two ways: gliding along the substratum or the water surface, and swimming freely. In our experiments, a reliable index of growth was easily obtained by counting individuals gliding on the liquid-glass surface. Culture vessels were chosen which facilitated such observations. Since nearly all media were turbid and, even with heavy growth, the turbidities were often of the same order of magnitude as the original medium, turbidometric measurement of growth was not feasible. Otherwise, the methods were those described for chrysomonads.[6]

The culture vessels were 125 × 20 mm. screw-capped tubes containing 10 ml. of medium. By setting a tube on the tilted stage of the microscope and focussing on the wall, direct counts could be made of the individuals in a low-power field (1.7 mm. in diameter). The tubes need not be opened—an important advantage. Counting was expedited, especially in dense cultures, by use of a Breed eyepiece micrometer disc which divides the field into ¼ths and ⅙ths. The large subjective error in this mode of expressing growth has several sources. Peranemas in newly inoculated cultures are nearly all at the bottom of the tubes where observation is hindered by the curvature of the wall. As they multiply, they ascend the walls until, in mature cultures, they are fairly evenly distributed from top to bottom. As cultures age, an increasing proportion of the population is at the bottom. Therefore, the cultures were inspected at least 3 times over a period of 4 weeks and tubes were not discarded until growth had clearly run its course. The error in counting different areas of a dense culture was about 25 per cent. Fields with more than 150 individuals were subjected to greater counting errors, since actively motile Peranemas might either be counted twice or elude counting altogether. The design of experiments took into account this crude measurement of growth; e.g., rather detailed concentration series were used for evaluating nutrients, and nearly every experiment incorporated controls recapitulating the salient findings of the preceding experiment.

Stock cultures were maintained in the following medium: liquid whole milk, 1.0 ml.; soil extract, 10.0 ml.; distilled water to 100 ml. The soil extract was prepared by autoclaving garden soil for 15–20 minutes with an equal weight of water, then filtering. Diluted whole milk or light cream alone supports good growth (100 individuals/field) within a month. Addition of the soil extract supplement permits heavier growth (200/field or 17,000/ml.) and the cultures last longer (2–3 months at 25–30 C.). Cultures deteriorate abruptly and therefore new cultures were started at two week intervals. The criterion of lowered viability was the increasing proportion of poorly motile, disintegrating, or thin individuals. At 18°C., cultures last 3–4 months and are correspondingly more slowly growing. Cultures placed at 6°C. die within 3 days.

Cultures for inocula were grown in 0.2–0.5 ml. of light cream/100 ml. of final medium. Not all tubes in the same batch may support growth. Inocula consisted of one drop of a "depleted" culture—one slightly past maximal growth—shaken vigorously to dislodge the Peranemas from the walls. These inocula did not carry over excessive amounts of fat- or water-soluble factors. In nearly all mature cultures, the medium becomes clear.

Media for stock and inoculation cultures were used at once, since upon storage there is a loss of ability to support growth as shown by an increasing proportion of unsuccessful transfers. It was convenient to keep on hand a 1:20 stock solution of cream with preservative added. This keeps for about a month at 6°C. More dilute solutions deteriorate with disproportionately greater rapidity. On the assumption that this deterioration was oxidative, several fat-soluble antioxidants were tried at concentrations up to 1.0 per cent, with various concentrations of cream, but no improvement was noted at nontoxic concentrations.

The following semi-synthetic medium supports heavy growth (200 individuals/field) when supplemented with light cream (0.03–0.05 ml./100 ml. final base). It represents a partial identification of the minimal requirements and of the sparing and stimulatory factors.

Results

Nearly all the information so far obtained on nutritional requirements is embodied in the basal medium shown in TABLE 1. This medium supports growth if supplemented with a small quantity of cream (0.03–0.05 ml./100 ml. final medium) or other complex materials. The concentration of cream required is reduced if water soluble materials, such as certain liver digests, are added.

The steps leading from the initial whole milk-soil extract medium to the semi-synthetic medium of TABLE 1 may be outlined:

(*1*) *Replacing soil extract.* Soil extract, an ultra-complex material, had to be eliminated as early as possible. One per cent whole milk, by itself, supported fair growth, but growth was greatly improved by the addition of a variety of commercial liver extracts as well as by soil extract.

(*2*) *Detection of lipid requirements.* In a simple basal medium containing glutamate, citrate, and the customary inorganic salts, whole milk was replaced by a smaller weight of cream. Growth in cream was good but was further improved by soil extract or any of several liver fractions. Skim milk, unlike whole milk, did not support growth. This pointed to a lipid requirement.

(*3*) *Detection of the sterol requirement.* A sharper separation of the water- and fat-soluble nutrients was desirable. To replace at least some of the water-soluble nutrients supplied by milk, the basal medium was supplemented with yeast autolysate (Albimi) + Wilson's "Liver L" (a tryptic digest of the fraction of liver insoluble in 70 per cent alcohol). Many experiments were performed aimed at identifying lipid requirements. TABLE 2 outlines the results of several. It should be noted that with lecithin as the only complex natural material in the medium, growth was negligible unless small amounts of cream, liver, or yeast were supplied.

(*4*) *Detection of amino acid requirements.* Concurrently with the lipid experiments, efforts were made to reduce the concentration of complex natural water-soluble materials. Principal reliance was placed on a mixture ("complete supplement") consisting of known vitamins, amino acids (as an acid hydrolysate of gelatin), and an alkaline hydrolysate of commercial yeast nucleic acid. "Complete supplement" did not completely replace complex natural materials such as yeast autolysate, liver preparations, milk, or cream. As improvements were effected in the general cultural conditions, the concentration of the complex materials required decreased. Amino-acid requirements now became apparent. Concentrations of gelatin hydrolysate as high as 0.6 per cent were needed for good growth. The way was now open for simul-

TABLE 1
FINAL BASAL MEDIUM

$(NH_4)_2H$ citrate	0.08 g.	DL-Tryptophan	0.01 g.
$CaCO_3$	0.005 g.	L-Glutamic acid	0.1 g.
KH_2PO_4	0.015 g.	DL-Methionine	0.025 g.
Mo (as NH_4 salt)	0.5 mg.	L-Cystine	0.01 g.
"Metals No. 42"	0.5 ml.	L-Histidine·HCl·H_2O	0.05 g.
$MgSO_4 \cdot 7H_2O$	0.04 g.	L-Arginine·HCl	0.05 g.
Na acetate·$3H_2O$	0.04 g.	DL-Aspartic acid	0.04 g.
Uracil	0.002 g.	"Amino acids No. 3"	4.0 ml.
Na guanylate	0.003 g.	Thiamine·HCl	0.2 mg.
Cholesterol	0.2 mg.	Riboflavin	0.01 mg.
Lecithin	0.006 g.	Cobalamin (B_{12})	0.05 μg.
		Thioctic acid (protogen)	250 units

Adjusted to pH 5.8–6.0, made to 100 ml. with distilled H_2O.

Notes on TABLE 1.

The basal medium was prepared in double strength and stored in the refrigerator with volatile preservative added.

1. Glutamic acid. The concentration employed is probably well in excess of the possible requirement. It also serves as a pH buffer.

2. Arginine, histidine, and aspartic acid. These are in the same category as glutamic acid. They are stimulatory and hence likely to prove essential when the basal medium becomes more precisely defined.

3. Methionine and cystine. Methionine proved essential. Sparing by cystine was not demonstrated.

4. Tryptophan. In early experiments an acid hydrolysis of gelatin was used instead of the amino acid mixture. Tryptophan is destroyed during hydrolysis and was therefore added separately. It is stimulatory in the present medium.

5. "Amino Acid No. 3." This supplement was prepared as a 20-fold concentrate. At the level of 4.0 ml. per 100 ml. final media, it yields the concentrations (%) shown.

DL-alanine	0.04	DL-leucine	0.004
L-arginine·HCl	0.04	DL-lysine·HCl	0.04
DL-aspartic acid	0.04	DL-methionine	0.0048
Na H glutamate	0.08	DL-phenylalanine	0.0032
glycine	0.04	L-proline	0.0032
L-histidine·HCl·H_2O	0.016	DL-serine	0.008
L-hydroxyproline	0.00048	DL-threonine	0.008
DL-isoleucine	0.004	L-tyrosine	0.0032
		DL-tryptophan	0.004
		DL-valine	0.004

pH adjusted to 7.0

6. $(NH_4)_2H$ citrate. Citrate acts as a buffering agent for essential metals, as pH buffer, and perhaps also to satisfy a possible need for a citric acid cycle component.

7. Metals. Prepared as described for chrysomonads.[6]

8. Uracil, guanylic, adenylic and cytidylic acids. These purine and pyrimidines eventually replaced the hydrolyzed yeast nucleic acid previously needed for good growth. They supported nearly full growth. Uracil and guanylic acid represented absolute requirements. Guanine and guanosine have not been tested. Cytidylic and adenosine-3-phosphoric acids were stimulatory.

9. Cholesterol-lecithin. A cholesterol requirement, whose specificity remains to be determined, was detected in early experiments on the fat-soluble factors. Soybean lecithin was found to be the most efficient dispersing agent for cholesterol. Cholesterol was dispensed from a stock solution in 1-chlorobutane. Lecithin was emulsified in water and added simultaneously with cholesterol. To obtain a fine suspension, the mixture was squirted into the basal medium through a 26-gauge syringe needle.

10. Thioctic acid. The synthetic compound, in the form of the *S*-benzyl thiuronium salt, was generously supplied by Doctors H. P. Broquist and E. L. Patterson of the Lederle Laboratories. Its essentiality has not been determined.

taneously identifying the effective components of the gelatin hydrolysate and for defining the properties of the unidentified factor (s) in the complex materials. Gelatin hydrolysate was replaceable by a mixture of amino acids predominantly synthetic in origin (See notes for TABLE 1).

(5) *Identification of purine and pyrimidine requirements.* The effective constituents of the "complete supplement" could now be dissected out. Omission of hydrolyzed yeast nucleic acid sharply curtailed growth. As mentioned in the discussion of TABLE 1, a combination of uracil, and guanylic, adenylic, and cytidylic acids replaced the hydrolysate.

TABLE 2

LIPID EXPERIMENTS

Basal Medium: citrate, glutamate, inorganic salts, yeast autolysate 0.025%, "Liver L" 0.003%, and gelatin hydrolysate 0.8%

		Lec.	C.	Lec. + C.
Tween 40	0	0	0	+
Tween 80	0	0	+	+
Na oleate	0	0		
Corn oil	0	0	±	
Wheat germ oil	±	0	0	
Cod liver oil	0	0	0	
Ethyl cellosolve	0	+		±
Glyceryl monooleate	0	±	±	+
Glycerol trioleate	±	+		+
NOPCO 10-A-0	0	+		+
Bile salts	±	+	±	+
Ox bile	±	+	±	+
Cholesterol	+	++	+	++
Lecithin	++	++	++	++
Light cream	+++	++++		++++

Lec. = lecithin; C. = cholesterol; Lec. + C. = lecithin + cholesterol

Scale: individuals per field

± = 5–10

+ = 10–40

++ = 40–80

+++ = 80–150

++++ = 150–200

Notes on TABLE 2

1. *Concentrations.* The concentration of nutrients were not indicated in TABLE 2 since it comprises several experiments. Concentrations ranged from very low amounts through several increments to toxicity.

2. *Lecithin.* Soybean lecithin, kindly supplied by The Glidden Company, allowed growth in the absence of cream. Without lecithin there was no growth. The crude lecithin at first used obviously contained a host of impurities. When a soybean lecithin preparation low in oil (Glidden lecithin "RG") was used, higher concentrations of this preparation were required. Since steroids are among the most tenacious contaminations of phospholipids,[3] cholesterol was tested in combination with the low-oil lecithin.

3. *Cholesterol.* In the presence of cholesterol (0.4 mg. per cent), 6.0 mg. per cent lecithin was required. The sterols in commercial soybean lecithin are sitosterols and stigmasterol. They may perhaps satisfy the steroid requirement.

4. *Light cream.* Growth in the crude lecithin or the lecithin-cholesterol combination did not equal the growth obtained with cream. The addition of lecithin-cholesterol to cream increased growth.

5. *NOPCO 10-0-A (National Oil Products Co.).* This is a non-ionic surface active agent of the polyoxyethylene-oleate type similar to Tween 80. Growth with the NOPCO product and the Tweens was poor.

(6) *Partial identification of vitamin requirements.* Vitamins were supplied at first as an arbitrary mixture whose effectiveness is shown in TABLE 3.

Since the liver preparation and cream had to be used in concentrations which masked requirements for certain known vitamins, and fractionation procedures had not yet been worked out, recourse was had to appropriate inactivation of these materials to demonstrate a few of the vitamin requirements. The lability of cobalamin to alkali was exploited in demonstrating its essentiality. Liver L was selected for this experiment because it contained an ample amount of cobalamin as surmised from the observation that media containing it did not require supplementation with vitamins to support growth.

TABLE 3

DEMONSTRATION OF REQUIREMENTS FOR WATER-SOLUBLE VITAMINS

Basal Medium: inorganic salts, citrate, glutamate, amino acids, uracil, guanylic, adenylic, cytidylic acids, lecithin 0.006%, cholesterol 0.1 mg.%, and gelatin hydrolysate 0.6%

				Vitamin mixture + thioctic acid*		
No additions	10	10	2	11	75	100
Liver L 0.001%	60	35	15	25	50	100
" " 0.003	70	100	65	10	60	120
" " 0.01	60	110	90	27	85	160
" " 0.03	70	145	120	7	85	200
Cream 0.001%	4	7	1	30	120	130
" 0.003	45	20	14	50	120	150
" 0.01	4	7	2	50	150	150
" 0.03	12	15	2	75	130	160
" 0.1	15	10	3	50	180	200

* Vitamin mixture used at level of 1.0 ml./100 ml. final medium, thioctic acid used at level of 1 unit/ml. final medium.

Results recorded after 2, 3, and 4 weeks.

Notes on TABLE 3

1. *Basal Medium.* The basal medium was supplemented with:

a) *Amino acids:* methionine, cystine, tryptophan, arginine, histidine, and aspartic acid.

b) *Gelatin hydrolysate:* prepared separately by autoclaving gelatin for about 8 hours in 5% H_2SO_4 at 115–120°C. The sulfuric acid was removed with $Ba(OH)_2$, activated carbon (Norit) added, and the $BaSO_4$ precipitate filtered off with the aid of diatomaceous earth ("Super-cel").

c) *"Vitamin Mix":* 1 ml. contains

p-Aminobenzoic acid	0.01 mg.	Biotin	0.5 µg.
Ca pantothenate	0.1 mg.	Citrovorum factor*	0.2 µg.
Choline chloride	0.5 mg.	Cobalamin	0.05 µg.
p-Hydroxybenzoic acid	0.01 mg.	Folic acid	2.5 µg.
Inositol	1.0 mg.	Riboflavin	5.0 µg.
Nicotinic acid	0.1 mg.		
Orotic acid	0.26 mg.		
Putrescine·2HCl	0.04 mg.		
Pyridoxine·HCl	0.04 mg.		
Pyridoxamine·2HCl	0.02 mg.		
Thiamine·HCl	0.2 mg.		
Thymine	0.8 mg.		

* As "Leucovorin" (Lederle) the synthetic $Ca\cdot 5H_2O$ salt. The concentration indicated is on the basis of the salt.

2. *Inoculum.* One drop of a 0.5 ml./100 ml. cream culture. The growth shown under the heading "no addition" indicates that the inoculum was not fully depleted.

3. *Cream.* No growth ensued with cream concentrations $\geqq$ than 0.1 ml./100 ml. unless the vitamin mixture was added. In the absence of cream, growth was further increased when Liver L was added along with the vitamins. As noted in TABLE 3, a sharper deficiency state for known vitamins was obtained with cream than with liver preparations.

Liver L was autoclaved for 1 hour at pH 10.0.[10] This procedure involved the risk of inactivating the unknown factor (s) in it, as well as the alkali-labile vitamins—thiamine, riboflavin, pyridoxamine, folic acid, and, to some extent, cobalamin. TABLE 4 shows the results of this experiment. Fortunately the unknown factor(s) was alkali-stable.

(7) *Identification of requirements for alkali-labile vitamins.* Alkali-treated liver supported good growth when supplemented with the 5 alkali-labile vitamins. They were added to a medium in which amino acids were supplied

as gelatin hydrolysate. Growth was poor when the gelatin hydrolysate in this set of experiments was replaced by pure amino acids. In another parallel set of experiments in which pure amino acids were used, low concentrations of untreated cream as the source of unknown factors supported growth when supplemented with the 5 alkali-labile vitamins. Cobalamin, thiamine, and riboflavin proved to be essential in this set of experiments. The essentiality of folic acid and pyridoxamine could not be demonstrated by this method, at least not at this stage.

TABLE 4

IDENTIFICATION OF ALKALI-LABILE VITAMINS

Basal Medium: inorganic salts, citrate, glutamate, amino acids, uracil, guanylic, adenylic, and cytidylic acids, lecithin 0.006%, cholesterol 0.1 mg.%, and gelatin hydrolysate 0.6%

	No addition			A			B		
No additions	5	2	0	0	0	0	0	0	0
Alk. Liver L 0.001%	5	3	1	70	60	50	—	—	—
" " " 0.003	13	4	1	70	65	60	75	70	70
" " " 0.01	25	5	3	50	55	60	75	80	90
" " " 0.03	30	30	2	65	75	50	80	80	90
" " " 0.1	30	30	2	65	30	30	75	35	30
Liver L 0.001%	40	20	15	60	50	30	75	75	60
" " 0.003	40	25	15	90	75	35	15	10	10
" " 0.01	30	30	35	100	90	60	95	90	60
" " 0.03	60	50	40	110	100	65	90	100	80
" " 0.1	60	80	100	120	100	100+	120	100	100+

"A": thiamin 0.2 mg. per cent, pyridoxamine 0.02 mg. per cent, riboflavin 0.01 mg. per cent folic acid 2.5 μg. per cent, cobalamin 0.05 μg. per cent.
"B": "Vitamin mixture 1.0 ml. per cent, thioctic acid 1 unit/ml.
Results recorded after 2, 3, and 4 weeks.

TABLE 5

GROWTH RESPONSE TO LIGHT CREAM WITH "FINAL" BASAL MEDIUM OF TABLE 1

	Days		
	21	24	26
No additions	10	20	0
Cream 0.001 ml./100	10	40	50
" 0.003 " "	10	20	50
" 0.01 " "	60	60	95
" 0.03 " "	50	90	125
" 0.1 " "	90	150	150+

The 3 vitamins demonstrated to be essential were incorporated into a new basal medium (TABLE 1) which allowed a smooth growth-response curve for cream (TABLE 5). That additional water-soluble nutrients are needed is rendered likely by the fact that the response to cream is more sensitive in the presence of "complete supplement" and deproteinized skim milk.[5]

Discussion

Peranema almost certainly shares some of the peculiarities of *Euglena* and *Astasia*, where an ostensibly simple pattern of nutrition has been described for the few strains studied in pure culture. But both kinds of euglenid are too superficially known to discern a common pattern of nutrition. For example, in *Peranema* the principal substrate, and in *Euglena gracilis* the system underlying the activation of glucose utilization by CO_2,[2] remain to be identified.

Along with the assay possibilities presented by the cobalamin requirement, perhaps most interest attaches to the lipid requirements. On the simple ground of poor diffusibility, lipids might be expected to be seldom required by osmotrophs. The lipid requirements of *Peranema* might be interpreted as evidence of a deep-seated adaptation to phagotrophy, as might also the cholesterol requirement of insects and the multiple lipid requirements of birds and mammals. A lipid requirement is not an infallible hallmark of phagotrophy; e.g., cholesterol, oleic acid, and vitamin K requirements are found in several nonphagotrophic protists, and, conversely, *Tetrahymena* and some phagotrophic chrysomonads, although readily ingesting lipid droplets, have no absolute lipid requirements. The steroid requirement of *P. trichophorum*, the need for at least one other lipid, the ready ingestion of fat droplets, and its predilection for surfaces, suggest that it is a useful guide in attacking some of the difficulties in supplying lipid nutrients which have impeded the development of synthetic media for the cells and tissues of vertebrates. This situation is discussed elsewhere[4] and is here briefly recapitulated: If the requirements of mammalian or avian cells mirror the requirements of the intact animal, then cell cultures must be supplied with (1) vitamins A, D, E, and K; (2) essential unsaturated fatty acids; perhaps (3) one or more unidentified lipids (no vertebrate has yet been grown on a synthetic ration with all lipid nutrients chemically defined); and perhaps, (4) with a modicum of fat as substrate to supplement glucose. How are these lipids to be made available? The outstanding effectiveness of lecithin as a dispersing agent for cholesterol for *Peranema* suggests trial of lecithin in cell cultures. Until the lecithin preparation used for *Peranema* is replaced with different and, if possible, purer compounds, other functions of lecithin for *Peranema* cannot be excluded. Another problem is how best to preserve essential lipids such as unsaturated fatty acids and vitamin E from oxidative destruction. Lecithin is a good antioxidant. The deterioration on storage of media for *Peranema* may result from the oxidation of a lipid growth factor similar to vitamin E or to unsaturated fatty acids. If so, *Peranema* would provide a convenient means of testing antioxidants for cell cultures.

Finally, since the role of the substratum in phagocytosis by leucocytes is a moot issue, elucidation of the connection between phagotrophy and need for substratum in *Peranema* may shed light on the behavior of leucocytes or, for that matter, of the ameboid cells found in all cellular animals from sponges and coelenterates to arthropods and chordates.

Summary

A strain of *Peranema trichophorum* in pure culture exhibited complex nutritional requirements. Among these were a steroid requirement satisfied by cholesterol plus lecithin; thiamine, riboflavin, and cobalamin (demonstrated via their alkali-lability); nucleic acid constituents (satisfied by uracil and guanylic, cytidylic, and adenylic acids); several amino acids; and unidentified fac-

tors found in liver digests and cream. The principal substrate was not identified.

As a phagotroph requiring lipid, *Peranema* holds promise as a test organism in exploring techniques for enhancing the availability of lipid nutrients. Its phagotrophy warrants further study to evaluate its application to assaying vitamins, notably cobalamin, in high-molecular combination.

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THE NUTRITION OF CARNIVOROUS PROTOZOA*

By Daniel M. Lilly
St. John's University, Brooklyn, New York

When the ciliate now known as *Tetrahymena pyriformis*[1] became available in pure culture it opened up a new field of study in the nutrition of many species of animal microorganisms that are either facultative or obligate carnivores. Appropriate techniques had already been developed to wash such organisms free of bacteria[2, 3, 4] and it appeared to be a simple procedure to culture them on a purely protozoan diet. In fact, the first references in the literature to such work[5, 6, 7] did not indicate any special difficulties and the possibilities of studying the nutrition of many carnivorous protozoa in two-membered cultures seemed practically unlimited. The fortunate and unique nutritional requirements of the genus *Tetrahymena* have received special attention by many investigators.[8, 9, 10] Our experience in feeding *Tetrahymena* to other protozoa serves to emphasize the difference between this well-known ciliate and some of the organisms which feed on it.

The first indication that *Tetrahymena* alone was not nutritionally adequate for some protozoa came from attempts to culture *Stylonychia pustulata*. In its natural environment, this common hypotrich feeds on such diverse organisms as bacteria, yeasts, algae, flagellates, and ciliates. It can, therefore, be considered practically omnivorous. In bacteria-free cultures, however, *Stylonychia* failed to grow on *Tetrahymena* unless a supplement was added to the medium.[11] This supplement could be obtained from yeast and from many plant materials, but apparently not from animal tissues. Another related hypotrich, *Pleurotricha lanceolata*, was found to require the same supplement. In both species of carnivorous protozoa, growth was proportional to the ciliate food rather than to the concentration of the supplement.[12]

Chemical properties of the supplement, such as its thermostability and solubility in aqueous but not in organic solvents, were soon determined. Much time was then spent in attempts to substitute known vitamins, particularly those of the vitamin B complex, but this approach was entirely unsuccessful.[12, 13] The natural occurrence of the factor in yeast suggested the B complex, yet its absence from liver and other animal tissues appeared to eliminate most of the known vitamins. The supplementary factor could be easily removed from yeast extract preparations by adsorption on charcoal. Elution was later accomplished with dilute ammoniacal ethanol, and a concentrate ten times as effective as the crude yeast extract was thus obtained.[13]

This success with adsorption and elution led naturally to chromatographic studies of the factor.[14] Two different methods of paper chromatography were employed, the ascending method of Williams and Kirby[15] and the chromatopile of Mitchell and Haskins.[16] The solvent used was ammoniacal ethanol in both cases. Since no color reaction was available for locating the position of the growth factor on the chromatogram, only biological tests could be used. Zones

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* Part of this research was aided by a grant from the National Science Foundation.

one centimeter in depth were taken from the paper, and individual aqueous extracts of each of these zones were tested for growth promoting activity for *Stylonychia*. The extracts from single zones gave negative results but, when extracts from two particular zones were combined, the growth of *Stylonychia* was equal to that in the control cultures containing yeast extract. The R_f values of the active zones compared favorably in both methods. The first interpretation of these results was that two distinct chemical factors might be involved. Recent work, however, with a different chromatographic method did not support this. The results are now believed to be due to such a distribution of the factor in the paper that zones of only one centimeter were too small to yield an effective concentration.

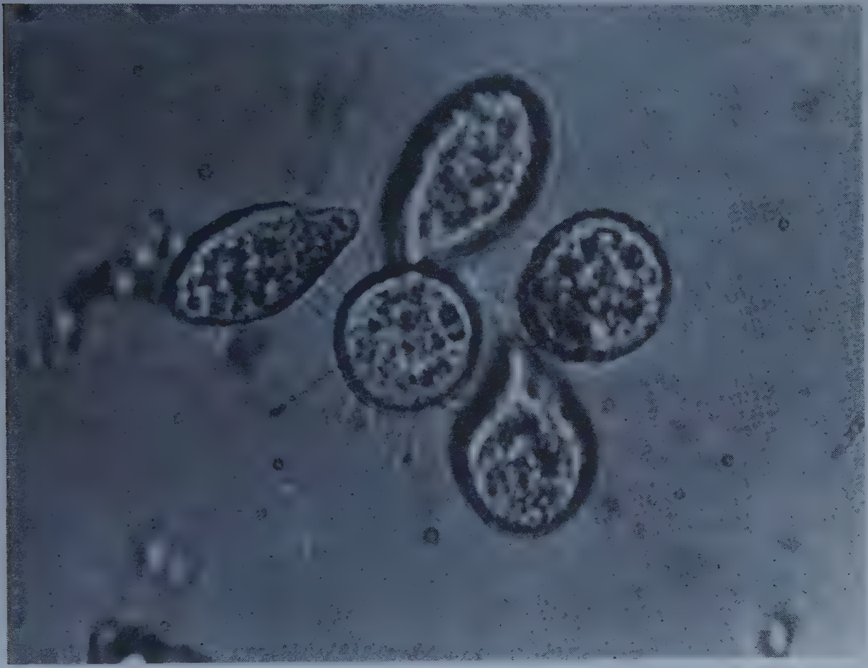

FIGURE 1. *Tokophrya infusionum* feeding on *Tetrahymena*. 1000 ×.

In the current method of concentrating and purifying the growth factor, Celite is used as an adsorbent in a chromatographic column. Celite is a diatomaceous material that is often considered inert, but it effectively removes the supplementary factor from yeast preparations. The adsorption from the crude extract transfers the factor to the column, which is then washed with neutral distilled water. The eluting mixture, one per cent ammonium hydroxide in 50 per cent ethanol, is passed through the column, and successive fractions are collected. Each fraction is evaporated to dryness, weighed, and redissolved in water to make stock solutions containing 100 micrograms of material per milliliter. Dilutions are then made and tested with cultures of *Stylonychia* to determine which fraction contains the greatest concentration of the supplementary factor. The most active concentrates thus far obtained show growth-promoting activity with as little as 0.1 microgram of material per milliliter.[17]

During the course of the purification of the supplementary factor, some preparations which were previously active later gave negative results. This inactivation was found to be due to exposure to light. This difficulty is now avoided by keeping both concentrates and cultures in the dark, but perhaps the photosensitivity of the factor may eventually aid in its chemical identification.

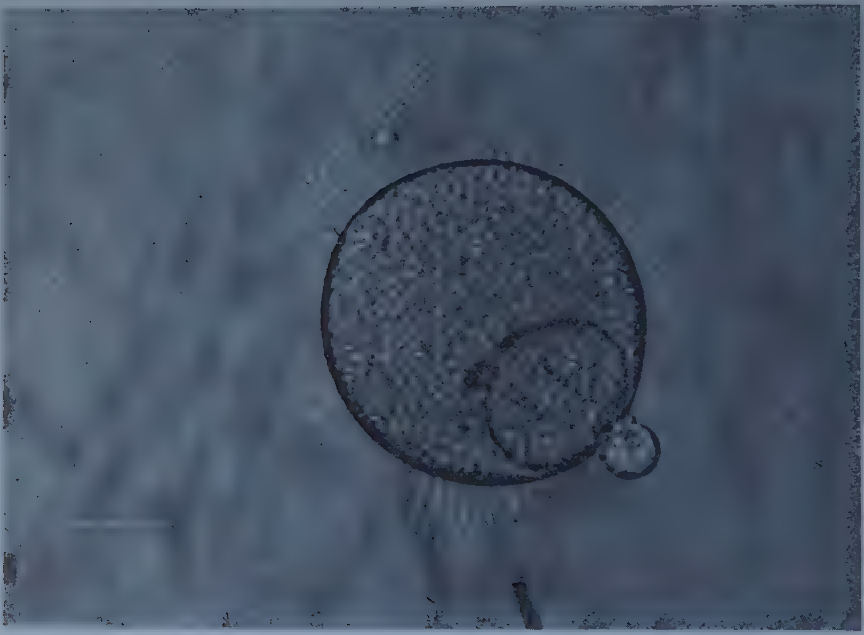

FIGURE 2. Normal reproduction of *T. infusionum* by internal budding. 1000 ×.

For comparative purposes, attempts were made at various times to obtain cultures of carnivorous protozoa other than *Stylonychia* and *Pleurotricha*. The simple screening method was to add *Tetrahymena pyriformis* and yeast to the samples brought in from natural waters and to observe the appearance and growth of suitable carnivores. The holotrichous ciliate now known as *Tetrahymena vorax* was discovered in this way and soon proved to be closely related to the ciliate on which it fed.[1, 3, 18] In another case, however, the hypotrich *Euplotes patella* fed on *Tetrahymena* only for a short time and apparently was unable to utilize the ciliate in its metabolism under the conditions tested. Eventually, this species was successfully cultured, but it was necessary to supply flagellates such as *Chilomonas* or *Polytoma*, as food. As long as the mixed bacterial flora from the original culture was still present, the *Euplotes* grew well but, as soon as one of the hypotrichs was washed free of bacteria, it failed to grow on the purely protozoan diet. This was in spite of the inclusion of yeast extract in medium on basis of experience with the other hypotrichs. Since the bacterial flora was apparently contributing some necessary factor, autoclaved suspensions of several kinds of bacteria isolated from the original culture were added to the medium. Two strains gave negative results, but the third enabled the *Euplotes* to grow at a slow rate. Later, the growth was greatly improved by the addition of liver extract. A satisfactory medium was eventually developed containing 0.06 per cent yeast extract, 0.007 per cent bacterial extract, and 0.015 per cent liver extract in addition to the flagellate food.[19]

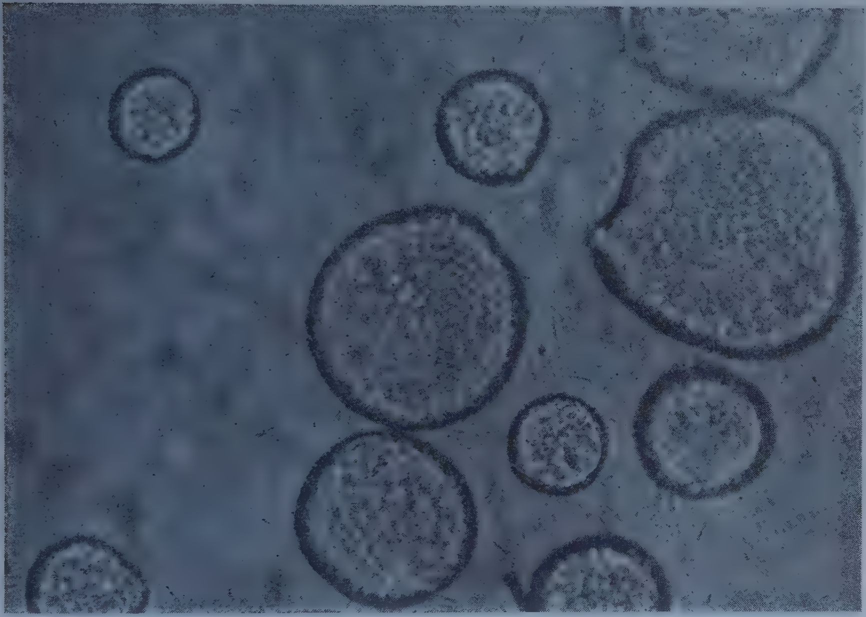

FIGURE 3. Normal and giant *T. infusionum*. 1000 ×.

This medium is so complicated that more work must be done on each of the crude extracts to determine the exact requirements of *Euplotes*. A new stimulus for resuming this investigation has been given by Fauré-Fremiet in his recent report that *Euplotes* has symbiotic bacteria within its protoplasm.[20] After treating the organisms with penicillin, he noted the disappearance of internal bodies resembling bacteria and a simultaneous cessation of growth. The cessation of growth could also be explained by the elimination of certain kinds of bacteria from the medium. As soon as new strains of *Euplotes* are obtained in bacteria-free culture, experiments are planned to test both explanations.

Among other organisms found in the continuing search for carnivorous protozoa were several species of suctorians, which proved useful in nutritional studies of a different type. Suctorians capture *Tetrahymena* or other ciliates and suck in their protoplasm through tentacles instead of ingesting the whole organism. A recent study by Kitching[21] indicates that the process is a true suction, although the exact mechanism has not yet been fully explained. Reproduction is not by fission, as in most protozoa, but by a process of budding involving the formation of a ciliated embryo which eventually swims away from the sessile parent. The young form later becomes sessile and develops tentacles to feed on suitable ciliates. It is interesting to note that different suctorian species utilize different kinds of ciliates, while apparently none of them feed on flagellates or ameboid organisms.

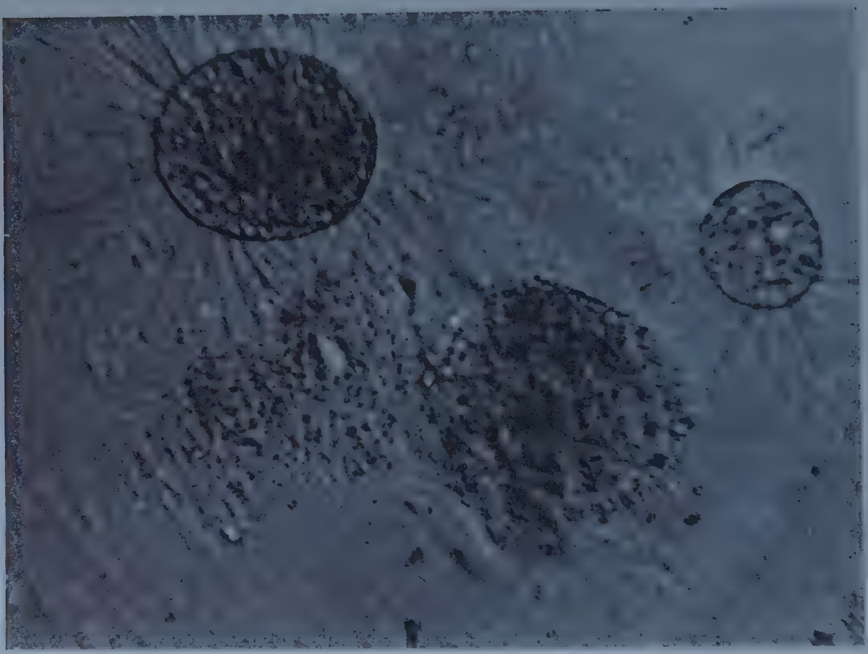

FIGURE 4. Cytolysis of giant form. Incipient giant and normal form shown for comparison. 1000 ×.

The suctorian *Tokophrya infusionum* was first established in bacteria-free cultures in 1940, but it was not until 1947 that a new strain was obtained for nutritional study. Collin[22, 23] studied this and other suctorian species forty years ago but, of course, not under controlled conditions. He reported that flourishing cultures could be maintained in the laboratory but that giantism and abnormal reproduction then became predominant with eventual loss of the cultures. In 1948, Rudzinska[24, 25] began working with one of our strains of *Tokophrya* and discovered that, when large numbers of *Tetrahymena* are supplied as food, the life span of the individual suctorian is shortened but, when food is restricted, the life span is prolonged. She also observed giantism and abnormal reproduction in cultures supplied with large numbers of ciliates. By carefully controlling the number of ciliates available as food, she was able to maintain cultures for several years.

Meanwhile, in our laboratory, a comparative study was undertaken on several species of suctorians.[26] *Tokophyrya infusionum* has so far proved to be the easiest suctorian to maintain on a diet of *Tetrahymena.* Yeast extract was found to improve growth and reduce the percentage of giantism and abnormal reproduction.[27] Guilcher,[28] working with a different suctorian species in France, has noted a similar improvement in cultures with yeast extract. When the yeast extract was omitted, growth did not cease immediately as in the case of the hypotrichs. There was, however, an increase in giantism and in abnormal reproduction and, after several transfers, the cultures usually died out. The growth factor concentrate effective for *Stylonychia* was not effective for *Tokophrya*, and it soon became apparent that different chemical entities were involved.

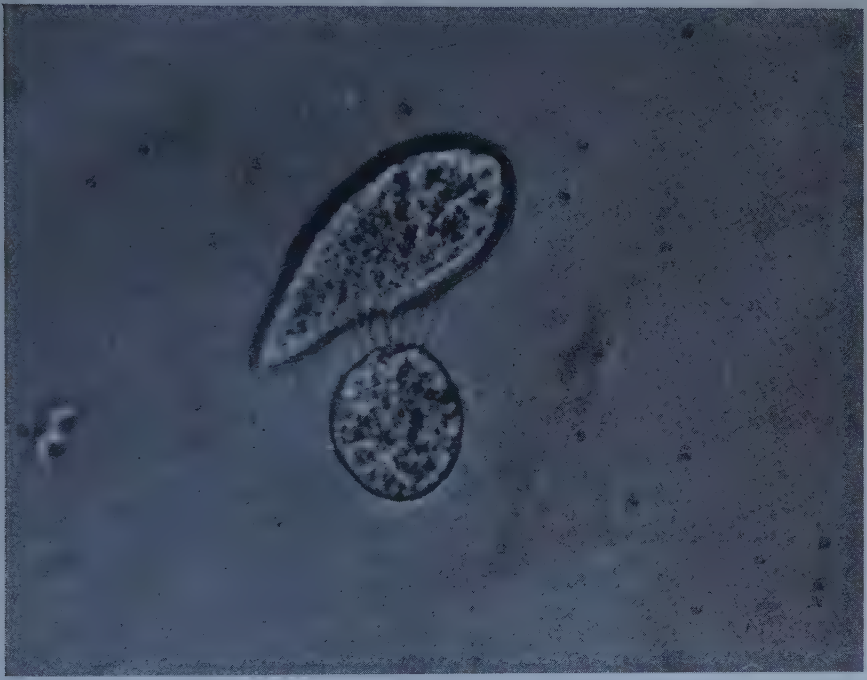

FIGURE 5. *Podophrya* feeding on *Tetrahymena.* 1000 ×.

Yeast nucleic acid in a concentration of 100 micrograms per milliliter proved to be just as effective as the crude yeast extract. Even better results in reducing the percentage of giantism and increasing the growth rate were obtained with a mixture of guanylic acid, adenylic acid, cytidylic acid, and uracil. A combination of guanylic acid and uracil was also effective, but individual purines and pyrimidines were without effect. Further work on the purine and pyrimidine metabolism of *Tokophrya infusionum* is now in progress. So far, it has not been possible to eliminate completely the occurrence of giant forms, and other factors may be involved.

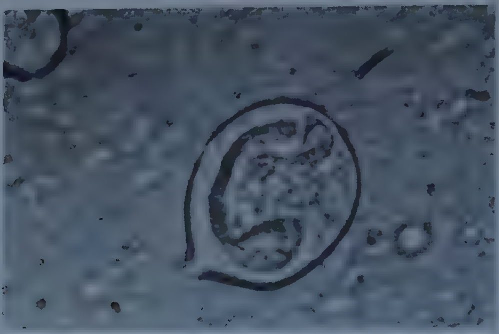

FIGURE 6. *Podophrya* starting to reproduce. 1000 ×.

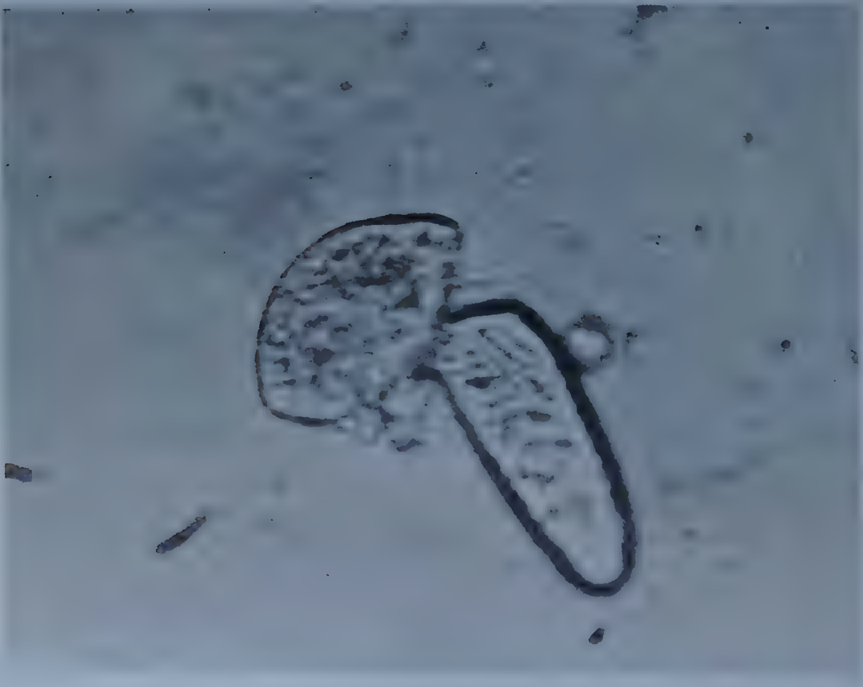

FIGURE 7. Normal ciliated external bud of *Podophrya*. 1000 ×.

One note on technique should be mentioned. Although stock cultures of *Tokophrya* thrive in test tubes, the growth is confined to a narrow zone just below the surface where most of the sessile forms attach themselves to the glass.

This makes observation and counting difficult for any sort of quantitative work. At present, experimental cultures are set up in sterile Syracuse dishes covered by an inverted watch glass and protected from contamination by cellophane bags. The watch glass prevents excessive evaporation, which would otherwise occur, but it can easily be moved aside for microscopic observation through the transparent cellophane. At the end of the experiment, when bacteria-free conditions need no longer be maintained, the final counts are made with the cellophane removed. Filling the dishes with medium and inoculation are accomplished either with long pipettes provided with rubber bulbs or with an automatic pipetting machine. Perhaps this technique may be useful for other protozoa that grow better in flat dishes than in tubes.

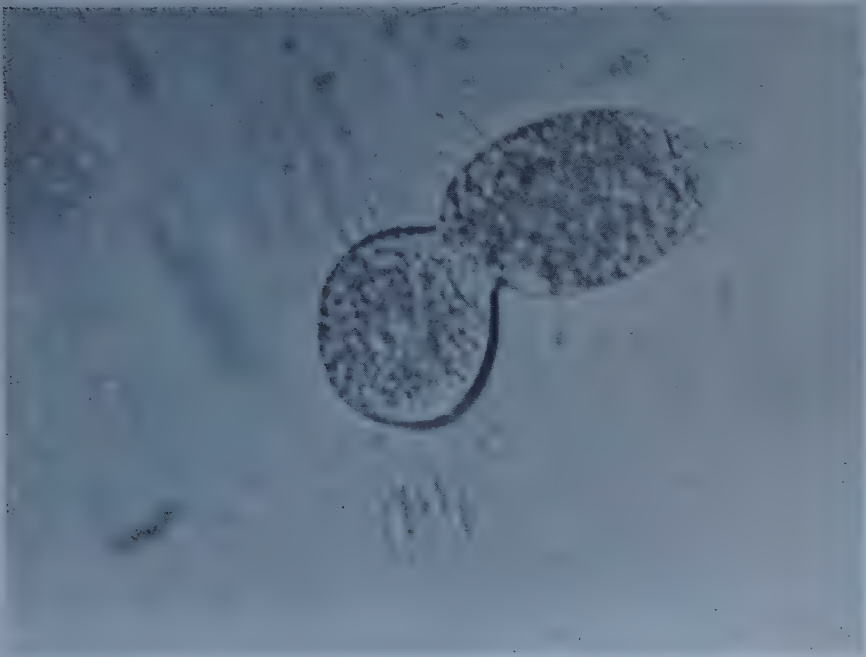

FIGURE 8. Abnormal reproduction showing tentacles on bud. 1000 X.

There are several other species of suctorians suitable for comparative studies. *Podophrya fixa* has a type of reproduction slightly different from that of *Tokophrya*, but it also feeds readily on *Tetrahymena* producing giants and reproduces abnormally when overfed.[26] Within the genus *Tokophrya* are several species that are more difficult to maintain in culture than *T. infusionum*. Preliminary work with *T. cyclopum* indicates that, when *Tetrahymena* is supplied as food, giantism and abnormal growth follow almost immediately but, if *Glaucoma scintillans* is substituted, better growth is obtained. There are also suctorians that feed on *Paramecium* but refuse *Tetrahymena*, and others that apparently feed exculsively on hypotrichs. Each species of suctorian may prove useful in studies on the biochemistry of the organisms on which they feed.

These studies on several different species of carnivorous protozoa bring new emphasis to problems long recognized in the nutrition of microorganisms. It is probably because of some of these problems that relatively few species of ciliates have been grown in the absence of other living organisms. In fact, the pure culture of any ciliate has always involved the solution of a growth-factor problem of some kind. With carnivorous organisms that will feed on *Tetrahymena* or other bacteria-free protozoa, it is possible to approach pure culture gradually, eliminating first the bacteria and solving whatever problems arise as a result of this step. Apparently, many ciliates have been so long associated with bacteria that, through changes in their enzyme systems, they can no longer synthesize certain substances needed in their metabolism. The work with hypotrichs indicates that growth factors not revealed by the requirements of other organisms may thus be identified and assigned a place in metabolic pathways. It must be remembered that practically all the vitamins were discovered by the study of deficiencies in various organisms. Hence, the prospect of different kinds of deficiencies in the numerous species of protozoa that remain to be investigated in pure culture raises the hope that many substances important in intermediary metabolism await discovery.

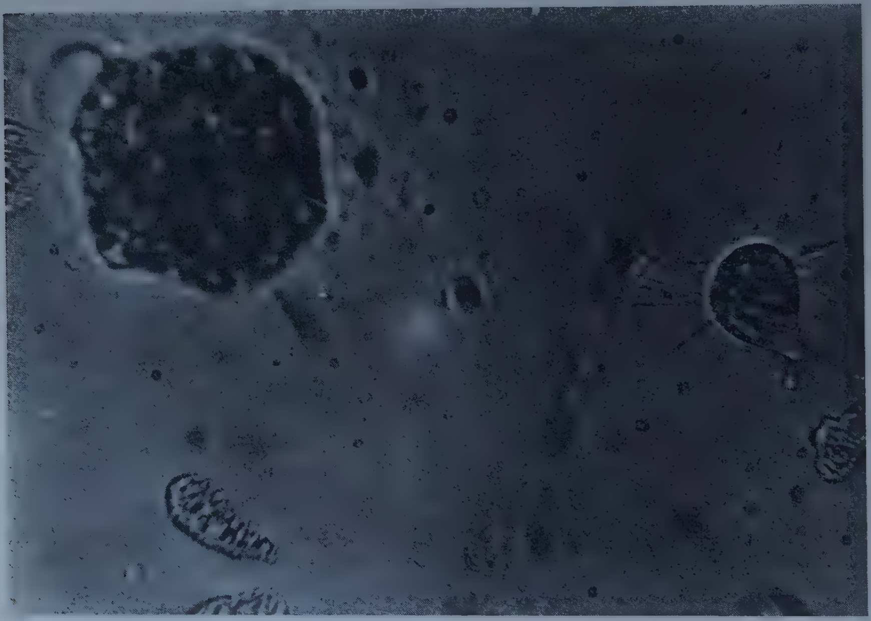

FIGURE 9. Normal and giant *Tokophrya cyclopum*. 1000 ×.

In attempting to advance from two-membered to pure cultures, however, there is less reason for optimism, since the second step in substituting chemical compounds for protozoan food often proves more difficult than the first. The use of killed organisms as food for the hypotrichs has, so far, been unsuccessful.

Even more remote at present is the probability of culturing suctoria on any food other than live ciliates. Perhaps after our immediate growth factor problems are solved, new ideas and new techniques will offer new hope for pure cultures.

In the work with the suctorians, the fact that the interference with reproduction is influenced by the amount of ciliate food available suggests some sort of imbalance of metabolites as the cause of the reproductive abnormalities. It is easily observed that the suctorians suck out first the cytoplasm of the ciliates and, when well fed, often release them without taking in nuclear components. Perhaps, this results in a nutritional imbalance which is corrected, at least partially, by the addition of purines and pyrimidines to the medium. There is still the objection that the effective purines and pyrimidines are those present in ribose nucleic acid, which is usually conceded to be in cytoplasm. It may be, however, that the quantitative distribution of these in the cytoplasm of *Tetrahymena* is of such a nature that the suctorians do not obtain the proper proportions when they take in only a cytoplasmic fraction of the ciliate.

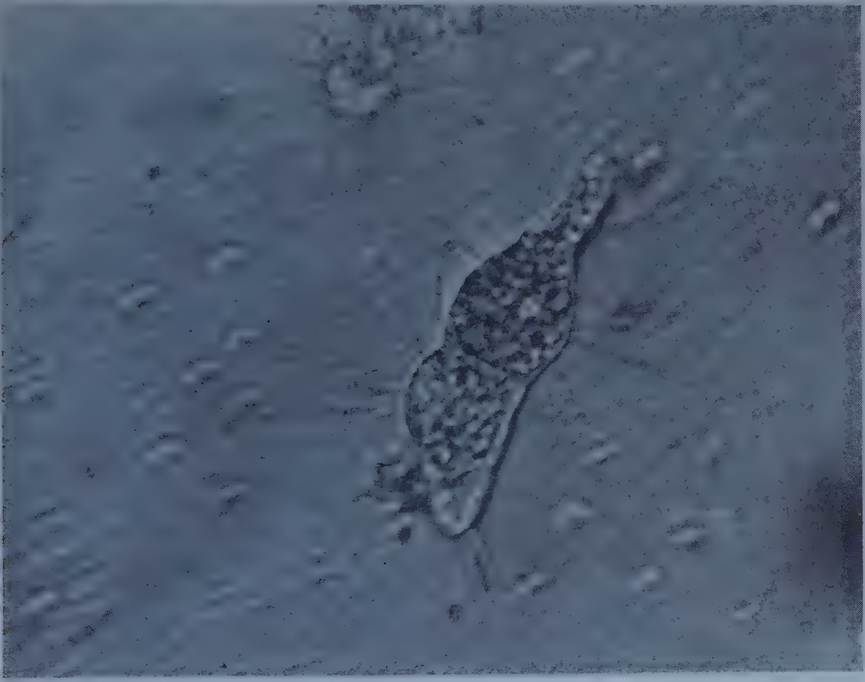

Figure 10. Conjugation in *T. cyclopum*. 1000 ×.

In using these suctorian protozoa for growth studies, a great advantage is the striking and rapid manifestation of the nutritional deficiency by giantism and abnormal reproduction. If more information can be obtained concerning the influence of nucleic acid metabolism on these abnormalities, perhaps this knowledge could be applied to the study of other abnormal cellular enlargement and proliferation, such as tumorous or cancerous growth in other animals.

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THE METABOLISM OF PROTOGEN IN *TETRAHYMENA*[*]

By Gerald R. Seaman
Department of Physiology, Medical Branch, University of Texas

The recognition by Snell and Broquist (1949) that protogen is identical with the acetate-replacing factor for *Lactobacillus casei* (Guirard *et al.*, 1946) and with the pyruvic oxidation factor for *Streptococcus faecalis* (O'Kane and Gunsalus, 1947) indicates that protogen is involved in the oxidation of pyruvate. Since acetate spares but does not replace the factor for the growth of *Tetrahymena* (Kidder *et al.*, 1950), it is evident that protogen is essential for reactions of pyruvate besides those leading to the production of acetate.

The initial reaction in the oxidation of pyruvate (reaction 1) includes electron transfer to DPN[†] and the transfer of the acetyl group to Co A. A similar electron transfer occurs in the oxidation of α-ketoglutarate with an accompanying transfer of the succinyl group to Co A (reaction 2).

$$\text{Pyruvate} + \text{Co A} + \text{DPN}_{\text{ox.}} \rightleftarrows \text{acetyl} \sim \text{Co A} + \text{CO}_2 + \text{DPN}_{\text{red.}} \qquad (1)$$

$$\alpha\text{-ketoglutarate} + \text{Co A} + \text{DPN}_{\text{ox.}} \rightleftarrows \text{succinyl} \sim \text{Co A} + \text{CO}_2 + \text{DPN}_{\text{red.}} \qquad (2)$$

The oxidations of both keto acids are analogous (Sanadi and Littlefield, 1952), and protogen may therefore be expected to perform the same function in the oxidation of α-ketoglutarate as it does in the oxidation of pyruvate. Not surprisingly, therefore, there occurs in protogen-deficient cultures of *Tetrahymena* an accumulation of α-ketoglutarate as well as of pyruvate (Dewey and Kidder, 1952).

A purified enzyme preparation showing both oxidative and acylative activity towards both pyruvate and α-ketoglutarate has been obtained from *Tetrahymena.*[‡] This preparation contains both specific oxidases and serves as convenient material for investigating the metabolic role of protogen. Washed ciliates obtained from yeast extract-peptone mass cultures (Seaman, 1953a) were concentrated by centrifugation and were ground with powdered quartz in a chilled mortar. The slurry was brought into solution with pH 7.6 phosphate buffer. The quartz was removed by gentle centrifugation and the supernatent was fractionated in the cold by isoelectric precipitation with 10 per cent acetic acid. The fraction precipitating between pH 5.4 and 6.3 was dissolved in phosphate buffer of pH 7.4 and was heated for 10 min. at 54°C. The suspension was then centrifuged briefly to remove heat inactivated protein. The supernatent exhibited a high activity toward both pyruvate and α-ketoglutarate that remained stable for as long as 4 months, when frozen, and stored at -20°C. The activity was also stable to lyophilization.

Oxidative activity of the two oxidases are readily measured by following the rate of reduction of 2,6-dichlorophenolindophenol (Sanadi and Littlefield, 1951). The acylative phase of the reactions was followed by measuring the appearance of acyl ~ Co A with the hydroxamic acid reaction (Lipmann and Tuttle, 1945). The rate of dye reduction and the amount of acylhydroxamic acid formed in the presence of either pyruvate or α-ketoglutarate is independent of the presence in the incubation mixture of cocarboxylase, DPN, or ATP. The necessary hydrogen acceptors and other cofactors are seemingly retained in these preparations. Cocarboxylase and DPN were nevertheless added to the incubation mixtures. Both the oxidative and acylative phases of the two oxidations show an absolute requirement for Mg^{++}. There is neither dye reduction nor formation of acyl ~ Co A in the absence of the ion. Co A and cysteine are also essential for maintaining acylative activity of both oxidases.

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[*] This investigation was aided by research grants from the U. S. Public Health Service and from the American Philosophical Society. Thanks are extended to Doctor E. L. R. Stokstad for generous supplies of synthetic protogen A (Bullock *et al.*, 1952), which is apparently the biologically active form (Seaman, 1953b). Appreciation is also expressed to Doctor Helmut Bienert for a sample of acetyl-Co A, and to Doctor F. M. Strong and to the Pabst Laboratories for a supply of highly purified Co A.

[†] The following abbreviations are used: Co A, coenzyme A; $\text{DPN}_{\text{oxid.}}$ and $\text{DPN}_{\text{red.}}$. oxidized and reduced diphosphopyridine nucleotide; ATP, adenosine triphosphate.

[‡] The S strain of *Tetrahymena pyriformis* (Corliss, 1952) was used.

The *Tetrahymena* enzymes contain much protogen, all of which is readily removed by shaking the suspension with a little alumina (Fisher "Adsorption, 80–200 mm."). After removal of the alumina by centrifugation, the supernatent is free of protogen, as determined by growth tests with *Tetrahymena*. The rate of dye reduction by the protogen-free preparations is identical with the rate shown by the untreated enzyme. The addition of pure protogen to either untreated or alumina-treated preparations had, as expected, no effect on the rate of reduction of the indophenol. On the other hand, protogen-free enzymes are completely inactive in carrying out the acylative reactions (TABLE 1). Acylative activity was completely restored by the addition of pure protogen to the acylation system. It is thus evident that protogen does not participate in the dye mediated hydrogen transfer, but protogen is essential for the acylation phase of the keto acid oxidations. The optimal concentration of protogen required for acylative activity is in the region of 20 units/ml. (FIGURE I).

TABLE 1

EFFECT OF PROTOGEN ON ACYL FORMATION*

Enzyme treatment	Acylhydroxamic acid formed	
	Pyruvic oxidase	α-Ketoglutaric oxidase
	μM	μM
None	0.96	0.83
None + 20 units/ml. protogen	1.10	0.94
Alumina	0.03	0.09
Alumina + 20 units/ml. protogen	1.04	0.89

* The complete incubation mixture contained 70 μg of cocarboxylase, 20 units of Co A, 10 μM $MgCl_2$, 100 μg of DPN, 10 μM of cysteine, 100 μM of $NaHCO_3$, 10 μM of pyruvate or α-ketoglutarate, 0.20 ml. of enzyme for pyruvate oxidation of 0.26 ml. for α-ketoglutarate oxidation in 1.0 ml. Incubation time 90 min. at 25°C.

Protogen, while essential for the transfer of acyl groups *to* Co A, is apparently unnecessary for the transfer of acyl groups *from* Co A. Protogen-free preparations of choline acetylase prepared from *Tetrahymena* form acetylcholine from acetyl ~ Co A and choline at the same rate as do preparations not treated with alumina. Choline acetylase was prepared from washed concentrates of the ciliate according to the method described by Nachmansohn *et al.* (1949) for use with rabbit brain. Experiments conducted with sulfanilamide acetylase prepared from duck liver (Kaplan and Lipmann, 1947) indicate likewise that acylation reactions are independent of protogen when acyl ~ CoA serves as the substrate.

Substrate balance experiments (TABLE 2) show that increasing the protogen concentration in incubation mixtures containing alumina-treated enzymes re-

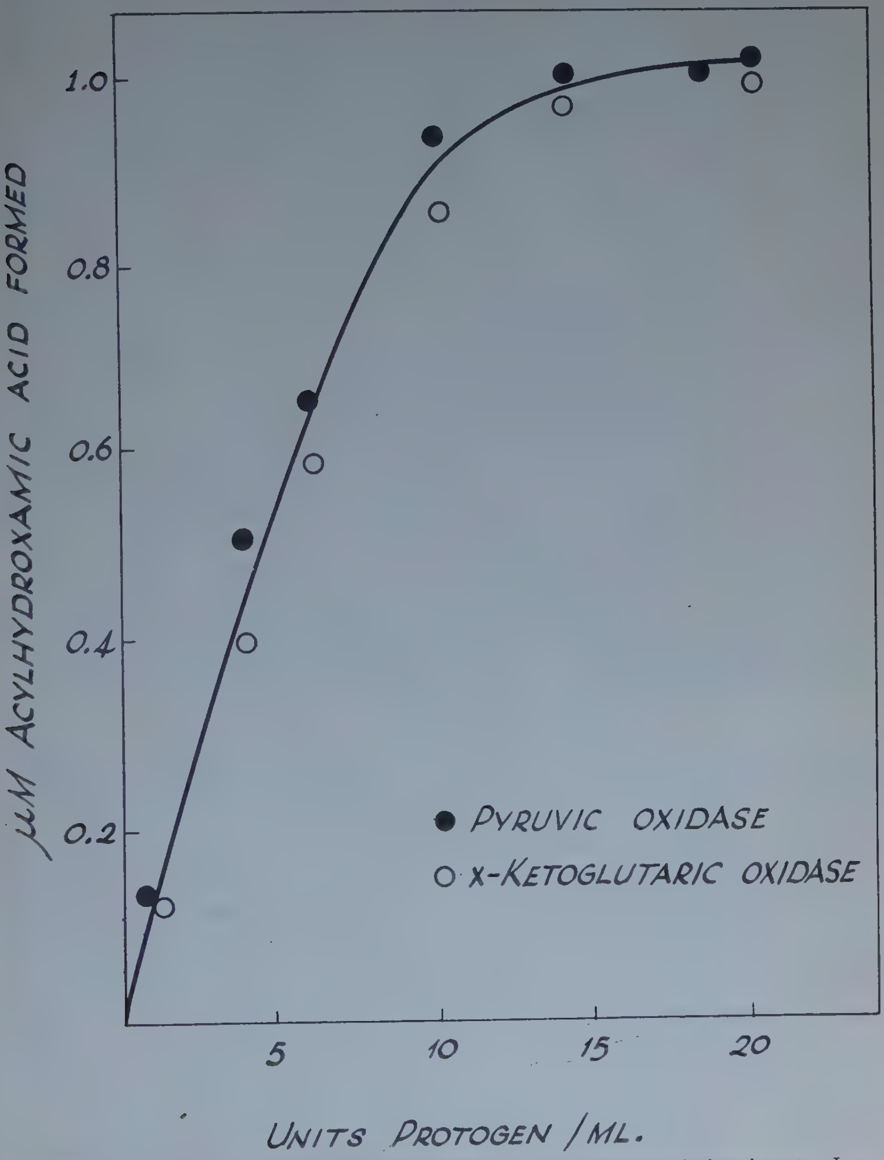

FIGURE 1. Requirement of protogen for formation of acyl ~ CoA from pyruvate and α-ketoglutarate. Incubation mixture was as described in TABLE 1. The mixture was incubated 90 min. at 25°C.

sults in the increased removal of keto acids. In like manner, the rate of acyl $\sim$ CoA formation and the rate of reduction of DPN is also increased. Formation of acetate and of succinate, the primary oxidation products of the respective keto acid transformations (Seaman, 1952a), is increased only slightly by the addition of protogen.

The finding that protogen is essential for transfer of the acyl group but is not required for electron transfer indicates that the first product of pyruvate oxidation is an activated C_2 fragment that either reacts with CoA to form acetyl $\sim$ CoA or is transformed to acetate. The direction of reaction of the activated fragment is dependent upon the presence of protogen, as indicated in TABLE 2. Oxidation of α-ketoglutarate is analogous in that the primary product is an activated C_4 fragment and the end products are succinyl $\sim$ Co A and succinate.

TABLE 2

EFFECT OF PROTOGEN ON KETO ACID FORMATION*

Protogen concentration	Pyruvic oxidase				α-Ketoglutaric oxidase			
	Substrate utilization	DPN reduction	Acetate formation	Acyl CoA formation	Substrate utilization	DPN reduction	Succinate formation	Acyl CoA formation
units/ml	μM	μM	μM	μM	μM	μM	μM	μM
0	0.34	0.30	0.34	0.02	0.31	0.33	0.30	0.00
1	0.41	0.37	0.32	0.09	0.36	0.35	0.36	0.04
5	0.64	0.59	0.36	0.30	0.58	0.51	0.31	0.26
10	0.90	0.92	0.36	0.54	0.88	0.82	0.38	0.46
20	1.12	1.00	0.42	0.68	1.05	0.99	0.46	0.55
30	1.07	1.00	0.39	0.64	0.99	1.03	0.40	0.58

* The incubation mixture contained 20 μM of $MgCl_2$, 200 μg of cocarboxylase, 50 μg of DPN, 60 units of Co A, 200 μM phosphate, pH 7.2, 10 μM of pyruvate or α-ketoglutarate, 10 μM of cysteine, and 0.2 ml. of *Tetrahymena* oxidase for pyruvate oxidation or 0.26 ml. for α-ketoglutarate oxidation in a volume of 3.0 ml. Enzyme preparation was treated with alumina. Keto acids were determined colorimetrically (Friedmann and Haugen, 1943); acetate and succinate were determined enzymatically (Soodak and Lipmann, 1948 and Krebs, 1937); DPN reduction was measured by following the reduction of added ferricyanide (Kolthoff and Menzel, 1929) in a parallel incubation mixture containing the above components with the exception that cysteine was omitted.

In addition to undergoing deacylation to acetate and succinate, acyl $\sim$ Co A serves as the acyl carrier for such acceptor systems as citrate (Stern and Ochoa, 1951), acetylcholine (Korey *et al.*,1951), acylated amines (Chou and Soodak, 1952), and pyrrols (Shemin and Wittenberg, 1951). It would be anticipated, therefore, that, in the growth of *Tetrahymena*, protogen may be spared by adding end products of these acyl transfers to culture media (Reed and DeBusk, 1952). Since many of these compounds are either unstable in solution or are commercially unavailable, at this time, succinate and citrate were chosen as the metabolites of choice to test for protogen-sparing activity. Dewey and Kidder (1952), however, have reported that the requirement for the vitamin could not be decreased by the addition of citrate or succinate. This lack of response may well be due to the testing of the metabolites in a medium at a pH well above the pK values of citric and succinic acids. As a consequence, the acids were in the dissociated form, in which form, they penetrate the cell membrane poorly or not at all (Seaman and Houlihan, 1950). When citric and succinic acids were added to washed, starved living cells at pH 5.3 (the lowest pH consistent with growth), both compounds elicited a definite increase in oxygen uptake.

Before carrying out sparing experiments with citrate and succinate, it was necessary to ascertain the optimal concentrations of protogen and of acetate necessary for ciliate growth at the low pH. Maximum protogen-sparing activity of acetate is obtained at 50 μg. of acetate/ml. At this concentration, the protogen requirement is satisfied by 0.1 unit/ml. of the vitamin. As frequently reported, concentrations of acetate in excess of that required for maximum sparing activity sharply inhibit growth. In these experiments at pH 5.3, the optimal concentration of acetate is much lower than at pH 7.4, the usual pH of culture media for the ciliate. Furthermore, as indicated in FIGURE 2, this lower concentration of acetate at the low pH has a greater sparing action than is observed at the higher pH. At pH 7.4, 500 μg./ml. of acetate are required to decrease the protogen requirement from 1.0 to 0.3 units/ml. At pH 5.3, 50 μg./ml. of acetate are capable of reducing the protogen required from 0.6 to 0.1 units/ml. The reason for this greater sparing activity of acetate at the lower pH value is obscure.

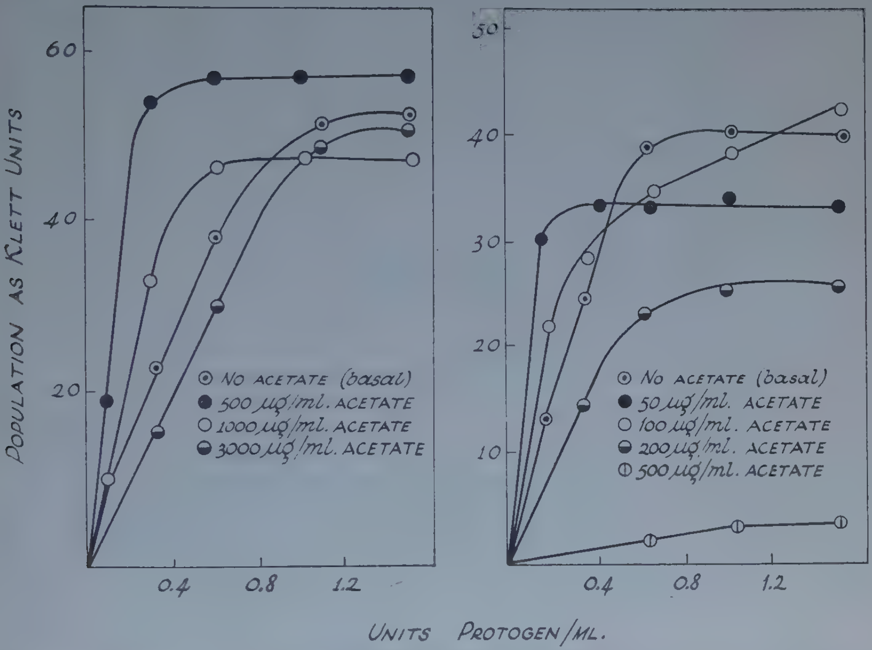

FIGURE 2. Sparing of protogen by acetate at pH 5.3 and at pH 7.4. The growth medium described by Elliott (1949) was used with the exceptions that the concentration of phosphates was altered, when necessary, to attain buffering action at the low pH, the recommended concentration of inorganic salts was doubled, and synthetic protogen was used.

The protogen requirement is decreased to 0.07 units/ml. by the addition of 10 μg./ml. of citrate to media at pH 5.3 containing 50 μg./ml. of acetate.

Higher concentrations of the tricarboxylic acid, as with acetate, are inhibitory. Addition of 25 μg. of succinate/ml. to media containing optimal concentrations of acetate and citrate results in a further lowering of the protogen requirement of *Tetrahymena*. In the presence of 50 μg. acetate/ml., 10 μg. of citrate/ml., and 25 μg. of succinate/ml., the protogen requirement is satisfied by only 0.03 units of protogen/ml. (FIGURE 3).

The pH of the medium, although initially adjusted to 5.3, gradually rises during growth to about 7.6. This increase in alkalinity probably results from ammonia formation. In any case, the pH of the culture early reaches a value too high for penetration of succinate and citrate. The sparing action of these metabolites is exerted, therefore, only during the early stages of growth.

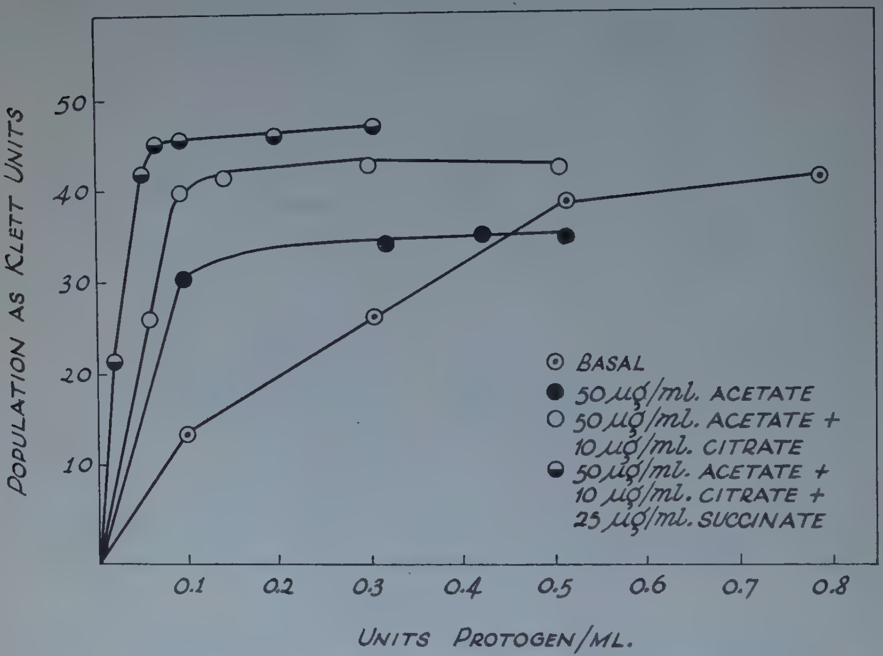

FIGURE 3. Sparing of protogen by acetate, succinate, and citrate. The conditions were as described in FIGURE 2.

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Discussion of the Paper

Virginia C. Dewey (*Department of Biology, Amherst College, Amherst, Mass.*): So far, it has been possible to demonstrate a sparing action on protogen in the growth of *Tetrahymena* by the use of acetate. Dr. Seaman has also shown an increased sparing action by the use of a combination of succinate and citrate with acetate. This we have been unable to demonstrate, although the difference in pH at which the experiments were carried out may explain the differing results.

In no case has complete replacement of protogen for the growth of *Tetrahymena* been possible. We have, therefore, been investigating other roles of protogen in the metabolism of the ciliate. Partition chromatography of the medium after growth of a culture deficient in protogen showed the accumulation of an unknown keto acid in addition to pyruvic and α-ketoglutaric acids. This acid reacts like α-ketoisocaproic acid, the deamination product of leucine. This suggests that one role of protogen is the oxidation of deaminated amino acid residues.

We have also found, recently, that protogen stimulates serine synthesis from glycine under certain conditions. When serine is absent and the protogen concentration in the medium is increased from one unit per ml. (the amount required for optimal growth) to ten units per ml., the amount of glycine required for a given amount of growth is reduced. Folic acid has an even greater stimulatory effect when its concentration is increased one-hundred-fold over that required for optimal growth. A tentative hypothesis to explain the stimulatory effect of protogen is that glycine, after deamination, yields the α-keto acid, glyoxylic acid. Under the influence of protogen glyoxylate is oxidized to CO_2 and "active formyl", which is then condensed through the mediation of folic acid, with another molecule of glycine to yield serine.

GROWTH REQUIREMENTS OF *PARAMECIUM AURELIA* VAR. 4, STOCK 51.7 SENSITIVES AND KILLERS IN AXENIC MEDIUM*

By W. J. van Wagtendonk, R. L. Conner,† C. A. Miller, and M. R. R. Rao
Department of Zoology, Indiana University, Bloomington, Indiana

The nutritional requirements of only a few ciliated protozoa have been investigated successfully, especially those relating to the single genus *Tetrahymena*.[1] Investigation of the nutritional requirements of protozoa is difficult due to the complexity of the natural habitat. Living bacteria often seem to be an absolute requirement. These bacteria may contribute labile complexes such as the "plasmoptyzate" factor for *Colpoda duodenaria*.[2, 3] Such factors are difficult to cope with, since they may be inactivated in the course of chemical separation, or they may be heat labile.

Paramecium aurelia is routinely grown on a water extract of dessicated lettuce inoculated with *Aerobacter aerogenes* 24 hours before use.[4] Lettuce or other plant extracts are essential since it is not possible to grow *P. aurelia* in a salt medium to which the bacteria have been added or in other synthetic media that support growth of *Aerobacter*.[5] In the lettuce medium, living bacteria are also essential. Neither the lettuce extract nor an autoclaved culture of *A. aerogenes* in lettuce extract will support growth of *P. aurelia*. This could be interpreted to mean that *P. aurelia* needs some heat-labile complexes for growth and that the organism has lost its capacity to satisfy these needs by synthesis.

It was subsequently found, however, that *P. aurelia* can be grown in an axenic medium consisting of heat-stable components.[6] This medium, sterilized by autoclaving, consisted of a 1:1 mixture of yeast extract (Basamin Busch) and a 24-hour-old culture of *A. aerogenes* in the lettuce extract. A heat-stable component from the yeast extract could replace a heat-labile factor present in the bacteria. This medium supported growth of *P. aurelia* at the rate of one to two fissions per day, which is considerably lower than can be obtained with nonaxenic culture methods (4–6 fissions per day). The low fission rate indicates that the heat-labile factor(s) contributed by the bacteria are stimulatory. The organisms could be maintained in this medium through many transfers.

P. aurelia was later established in a medium developed by W. H. Johnson for the axenic culture of *Paramecium multimicronucleatum*.[7] This consisted of pressed-yeast juice and proteose peptone. The pressed-yeast juice was found unsatisfactory for further fractionation and contained heat-labile components. The medium had one advantage, however, in that it was free of debris. From this, a medium has been developed that is composed of heat-stable components and is free of cellular debris.

The composition is given in TABLE 1. The medium consists of (A) a salt

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† Predoctoral Fellow of the National Science Foundation.

solution, to which are added aseptically sterile solutions of (B) a mixture of the water-soluble vitamins; (C) a dialyzed yeast extract; (D) 0.8 per cent proteose peptone; (E) a factor isolated from lemon juice; and (F) guanylic and cytidylic acid.

The yeast extract is prepared from Fleischmann's bakers' yeast, which has been dried at 65°C. An 1:4 (w/v) suspension of the dried yeast is autoclaved for 20 minutes at 15 pounds pressure, centrifuged, and filtered through a Seitz filter pad for clarification. The filtrate is dialyzed at 4°C. for 96 hours against running distilled water. The contents of the dialysis bag are adjusted with NaOH to pH 7.0 and sterilized by autoclaving. This preparation is added to the salt solution so that the final concentration of yeast solids is 0.75 mg. per milliliter.

Paramecium aurelia, var. 4, stock 51.7 (sensitive), freed from bacteria either by penicillin treatment[6] or by Parpart's washing technique,[8] will multiply in this medium at a rate of 2–3 fissions per day, through successive four-day transfers. The organisms have been carried in this medium for over a year.

TABLE 1

COMPOSITION OF THE AXENIC STOCK MEDIUM

(A) *Salt solution:*		(B) *Vitamins* (final concentration per ml.):	
$NaCl$	150 mg.	Thiamine	5 μg.
KCl	25 mg.	Riboflavin	2.5 μg.
$CaCl_2 \cdot 2H_2O$	25 mg.	Niacin	18 μg.
$MgSO_4 \cdot 7H_2O$	50 mg.	Ca pantothenate	5 μg.
K_2HPO_4	100 mg.	Pyridoxine	5 μg.
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	5 mg.	Pyridoxal	2.5 μg.
Water	1 liter	Pyridoxamine	2.5 μg.
(C) *Yeast extract*	0.75 mg./ml.	Folic acid	2.5 μg.
(D) *Proteose peptone*	8 mg./ml	Biotin	1.25 μg.
(E) *Lemon factor*	200 mμg./ml.	Choline	5 μg.
(F) *Guanylic aicd*	50 μg./ml.		
Cytidylic acid	50 μg./ml.		

The dialyzed yeast extract, the proteose peptone, the B-vitamins and the factor from lemon juice are essential. No growth of *P. aurelia* takes place when any one of the components is omitted. In an attempt to obtain a vitamin-free medium, a nondialyzable fraction of proteose peptone was prepared. This preparation was not consistent, however, in promoting growth in consecutive transfers. The use of the nondialyzable fraction of proteose peptone was therefore discontinued.

This medium has been used to establish some of the vitamin requirements of this stock of *P. aurelia*. The technique of single omissions from the mixture of water-soluble vitamins was used. It must be emphasized that only failure of growth, as a result of the omission of a vitamin from the mixture, indicates a requirement for this particular vitamin. Limited growth or normal multiplication of the organisms when a vitamin is omitted does not necessarily indicate that this vitamin can be synthesized by *Paramecium*. In these cases, the possibility exists that the vitamin had not been completely removed from the yeast, or that it is present in the proteose peptone in sufficient quantities to allow growth of the organism. Thus, thiamine, riboflavin, and folic acid have been demonstrated to be essential metabolites for *P. aurelia*. The evidence is given in FIGURE 1. The first transfer into the medium deficient in thiamine

TABLE 2

THE ESSENTIALITY OF THE COMPONENTS OF THE AXENIC MEDIUM

Yeast	Proteose peptone	Lemon factor	Nondialyzable yeast	B vitamins	Growth
+	+	+			+
	+	+			−
+	+				−
+		+			−
	+	+	+		−
	+		+	+	−
	+	+		+	−
		+	+	+	−
	+	+	+	+	+

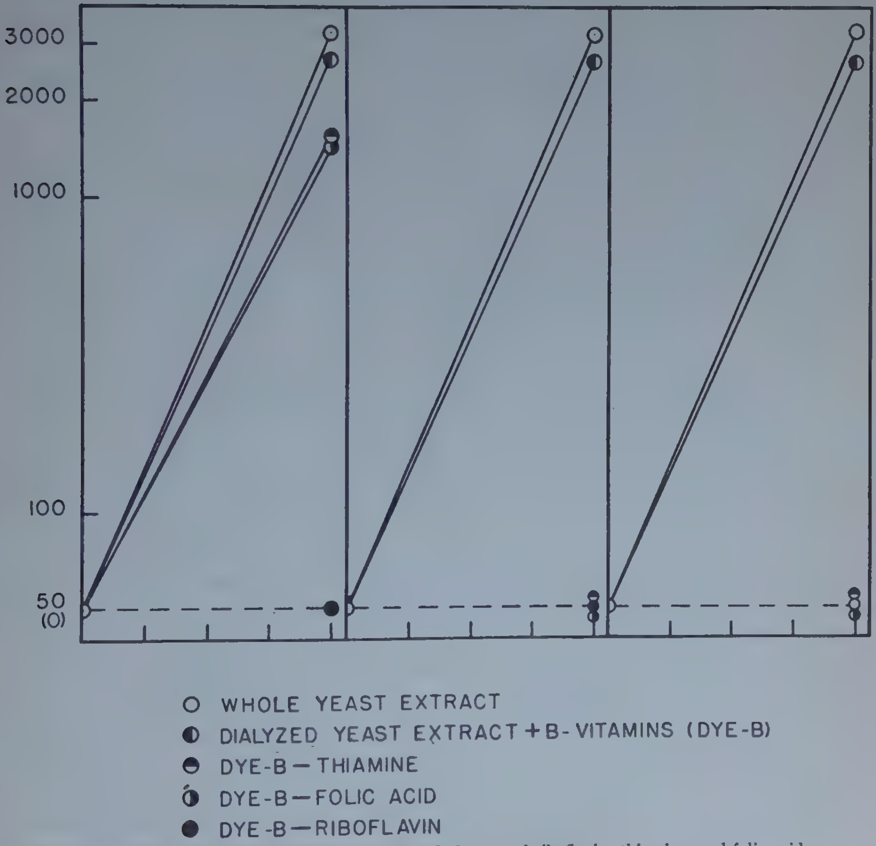


FIGURE 1. Growth of *P. aurelia* in the presence and absence of riboflavin, thiamine, and folic acid.

or folic acid shows reduced growth. In the second transfer, the animals do not multiply. Apparently, some carry-over of thiamine and folic acid permitted the animals to go through several fissions in the first transfer. Animals that have been transferred into the medium deficient in riboflavin do not multiply and eventually die. It is apparent from the growth curves that the nondialyzable yeast extract plus the B-vitamins, for all practical purposes, can replace the whole yeast extract.

It has not been possible to grow *P. aurelia* in a medium devoid of plant material. It is, therefore, evident that these extracts contain essential growth factors for this organism. The activity of several raw materials is given in TABLE 3. No tested animal sources exhibited growth-promoting activity. It is not known whether the activity demonstrated by the different plant extracts is due to the same factor.

Lemon concentrate was the most active source and was selected for the extraction and purification of the growth factor. The extraction procedure included precipitation of the active fraction with acetone, extraction of the active material from the precipitate with hot alcohol, phase distribution, and chromatography.[9] A crystalline material with the chemical and biological characteristics listed in TABLE 4 was isolated.

TABLE 3
RELATIVE ACTIVITY OF VARIOUS RAW MATERIALS
(Activity of cerophyl extract = 1)

	Relative activity		Relative activity
Cerophyl extract*	1	Lemon peel infusion†	0
Water extract of dessicated lettuce (Difco)	3	Wilson liver extract	0
Water extract of baked lettuce	4	Proteose peptone	0
Orange juice (fresh)	10	Bacto tryptone	0
Lemon juice (fresh)	15	Casamino acids	0
Lemon concentrate†	60		

* Obtained from the Cerophyl Laboratories through the courtesy of Doctor G. A. Köhler.
† Obtained from the California Fruit Growers Exchange through the courtesy of Doctor W. E. Baier.

The chemical analysis and the color reactions exhibited by this compound would place it in the class of the steroids. Several sterols were therefore tested for growth promoting activity. Cholesterol was inactive, stigmasterol (or its acetate), and a sample of tall-oil sterols (principally β-sitosterol) showed activity. The infrared spectrum of the compound isolated from the lemon juice was compared with those of cholesterol, stigmasterol, and the tall-oil sterols.

It can be seen from FIGURES 2 and 3 that the infrared spectrum of the growth factor for *P. aurelia* is similar to those of stigmasterol, cholesterol, and the tall-oil sterols. The infrared spectra of the chloroform solutions of recrystallized tall-oil sterols and of the growth factor for *Paramecium* are identical in wave length and intensity from 2.6 to 9.6 μ. Small differences in the range 9.6–12 μ include a weak unresolved band at about 9.65 μ and weak underlying absorption between 10.2 and 10.5 μ in the spectrum of the growth factor.

There are three distinct differences between the spectrum of the factor and that of cholesterol: (1) Although both compounds have a weak band at 5.99 μ, the factor has an additional band at 6.24 μ. (2) The ratio of intensities of hydrogen-bonded OH to free OH is distinctly greater in the factor than in cholesterol. (3) The intensity of the band at 9.52 μ is about 10 per cent greater in cholesterol than in the factor. These differences could be explained by postulating a different orientation of the hydroxyl and/or a different form of ring A (a boat rather than a chair form). The latter difference might explain the activation of a "second" stretching made of the Δ^5 double bond (6.24 μ). No new band appears in the C=C bending region in the factor or the tall-oil sample as it does in stigmasterol at 10.26 μ.

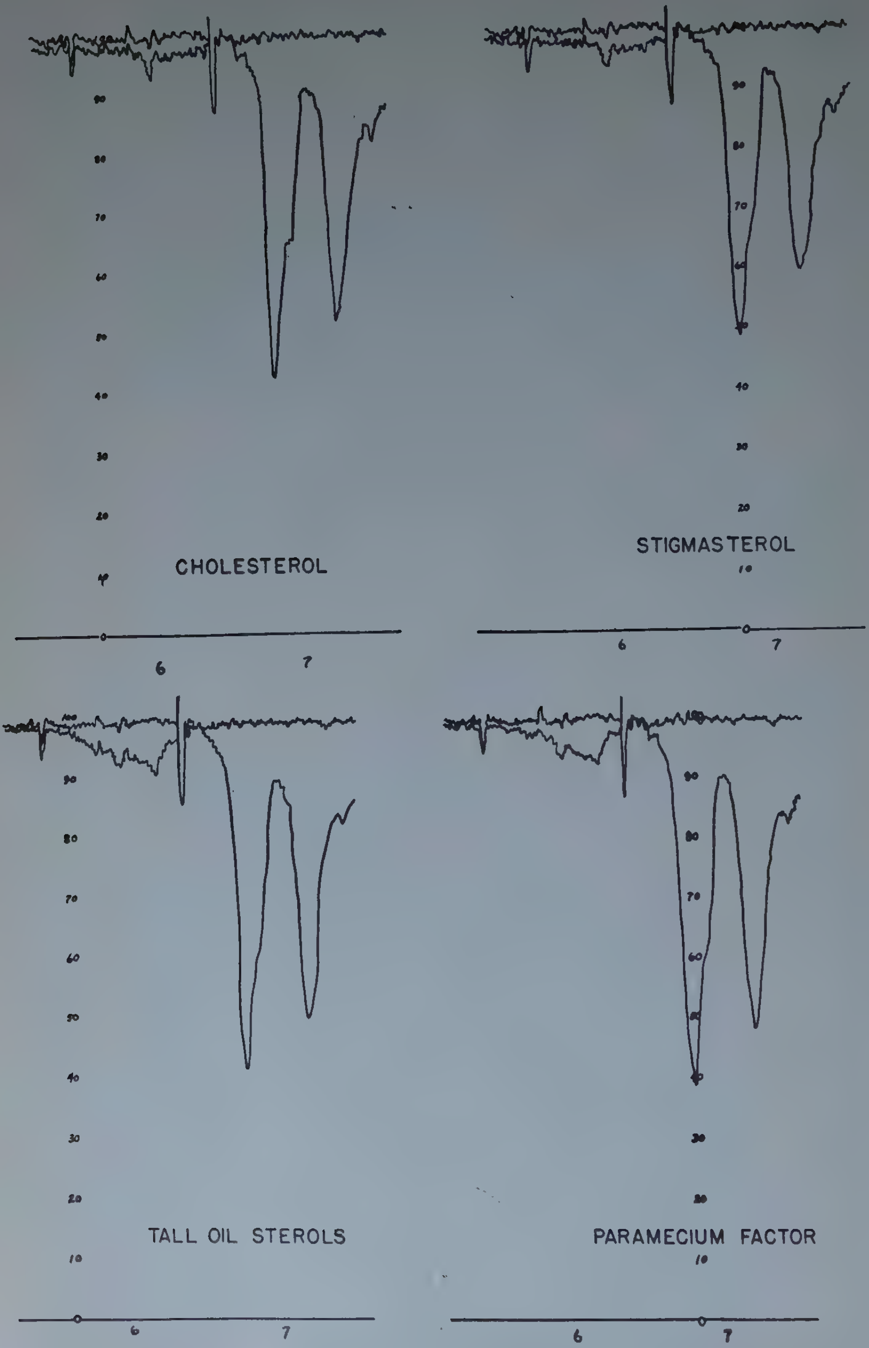
Because of the close similarity of the infrared spectra and because of the fact that the melting point of our compound is identical with that of β-sitosterol

TABLE 4

CHEMICAL AND BIOLOGICAL CHARACTERISTICS OF THE LEMON-JUICE FACTOR.

		C	H	
Melting point (uncorrected)				136.5–137.5°C.
Carbon and hydrogen analysis:				
Lemon factor		83.60	12.32	
Calculated for: $C_{27}H_{46}O$		83.80	11.91	
$C_{29}H_{48}O$		84.46	11.65	
$C_{29}H_{50}O$		83.92	12.07	
Color tests:				
Liebermann-Burchard	+			
Salkowski	+			
Rosenheim	−			
Biological activity:				
The compound promotes growth through successive transfers in a concentration of at least 50 mμg. per ml.				

(22:23-dihydrostigmasterol), we tentatively conclude that the growth factor isolated from lemon juice is closely related to, or identical with β-sitosterol. The role of cholesterol and other related sterols as growth factors was first demonstrated by Cailleau for *Trichomonas columbae.*[10] Cholesterol might fulfill a true metabolic requirement in this group of protozoa, since *Eutrichomastix colubrorum*, in axenic culture,[11] and *Trichomonas batrachorum*[12] and *Trichomonas foetus*,[13] in nonaxenic cultures, also require cholesterol. The only other sterol-requiring microorganisms that have been reported are *E. histolytica*[14] and a group of organisms of the pleuropneumonia group,[15] which require cholesterol. Vishniac and Watson have demonstrated that *Labyrinthula vitellina* var. *pacifica* also requires a steroid as growth factor.[16] Their results indicate that cholesterol, cholestenone, fucosterol, and β-sitosterol are active for this organism. Although our work on the steroid requirements for *P. aurelia* is still in its preliminary stages, some interesting comparisons can be made. Cholesterol acts as a growth factor for several of the protozoa, but it is completely inactive for *P. aurelia.* It appears that simple substitution at C_{24} imparts activity to the steroid molecule. There are also indications that the presence or absence of double bonds at certain positions influences the activity.


CHOLESTEROL

STIGMASTEROL

TALL OIL STEROLS

PARAMECIUM FACTOR

FIGURE 2. Infrared absorption of the growth factor, cholesterol, stigmasterol, and tall oil sterols in the region of 5–7 μ.

It seems unlikely that the specific activity of the steroids can be explained along the lines suggested by Gaines and Totter for the action of dehydroepiandrosterone in replacing leucovorin and pteroylglutamic acid (folic acid) for *Leuconostoc citrovorum* and *Streptococcus faecalis*,[17] since folic acid was present in our basal medium.

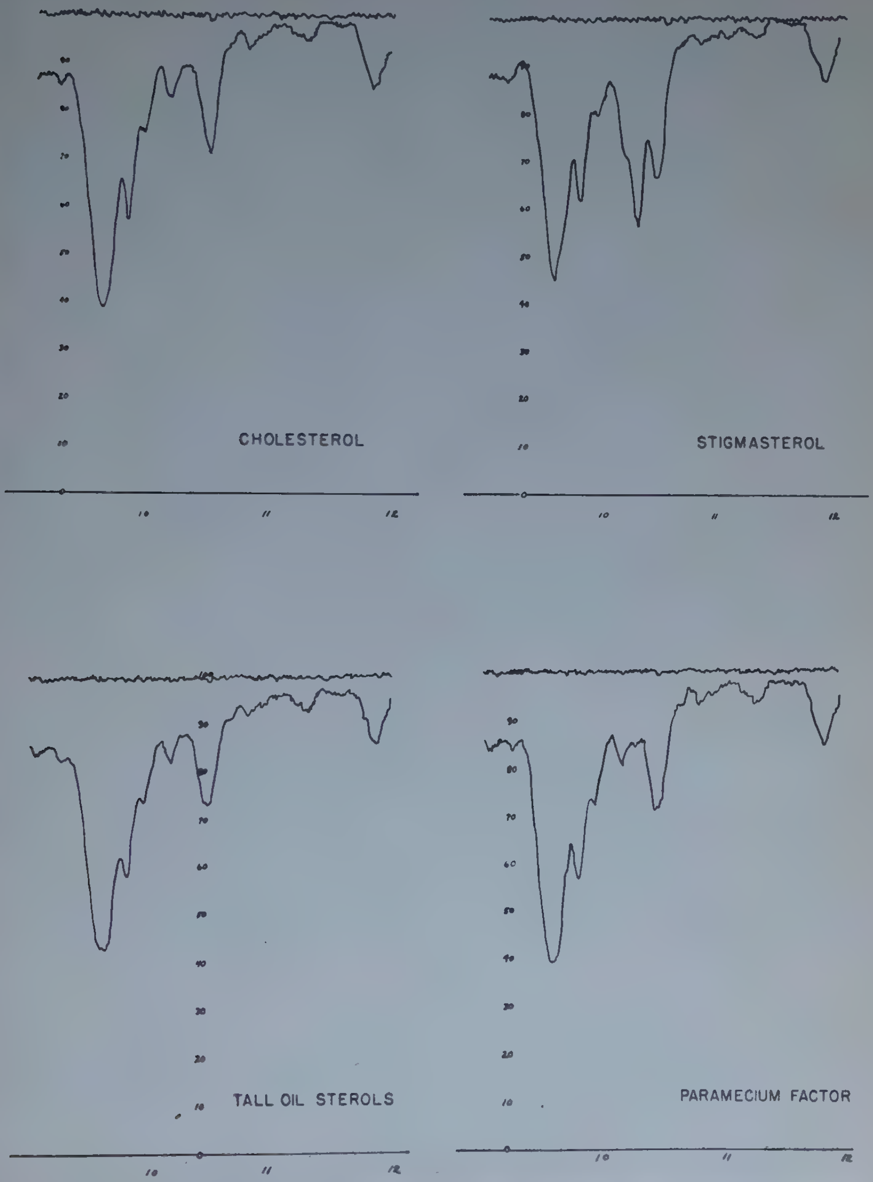


FIGURE 3. Infrared absorption of the growth factor cholesterol, stigmasterol and tall oil sterols in the region of 9–12 μ.

The *Paramecia* used in these investigations were all of the killer genotype and the sensitive phenotype. Animals of the sensitive phenotype differ from the killers by the absence in the cytoplasm of a particulate cytoplasmic component, kappa. The particular strain from which the sensitive strain was derived contains normally from 400–1000 kappa particles per cell. This amount is dependent upon the feeding conditions and is different in the various stages of the life cycle.[19] Killers can be freed of the cytoplasmic component by growing them at 33.8°C. At this temperature, kappa loses its capacity for multiplication, and is consequently lost by dilution. The resulting animals are sensitive to the killing action by paramecin, a toxic substance secreted by the killers. The presence of kappa in the cell influences the metabolism of the cell.[18] The axenic medium that was developed for the sensitives is not suitable for the killers. Killer animals freed from bacteria by Parpart's washing technique will go through one to four fissions and die. Similar observations were made with killer animals that had been sterilized with penicillin. Occasionally, a few penicillin-treated killers grew in the axenic medium. Staining of these animals revealed, however, that they had lost the kappa particles. They were also susceptible to the killing action when mixed with killer animals, and they did not kill when mixed with sensitive animals. The loss of kappa was due to the penicillin treatment. Killer animals with a normal kappa count that were exposed to penicillin G (3200 units per ml.) for 48 hours would lose up to 80 per cent of the original number of kappa particles. Longer exposure (up to 96 hours) resulted in a complete loss of kappa. These animals had become sensitives and were now able to multiply in the axenic medium. The inability of the axenic medium to support the growth of killers indicates that the presence of the cytoplasmic factor in the cell alters the growth requirements for *P. aurelia*. These requirements are fulfilled by the usual bacterized stock medium but not by the axenic medium in its present form.

Summary

Paramecium aurelia, var. 4, stock 51.7 (s) has been established in an axenic medium. In addition to a dialyzed yeast preparation and proteose peptone, riboflavin, folic acid, and thiamine were found to be essential nutrilites. The requirements of the other members of the group of the water-soluble vitamins could not yet be established. A steroid fraction obtained from lemon juice was also found to be required. Infrared-absorption spectra and biological-activity tests indicate that this sterol is closely related to β-sitosterol. Cholesterol is inactive. The axenic medium does not support the growth of killers.

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THE ISOLATION AND CULTURE OF OPSIMORPHIC ORGANISMS. I. OCCURRENCE AND ISOLATION OF OPSIMORPHIC ORGANISMS FROM SOIL AND CULTURE OF ACRASIEAE ON A STANDARD MEDIUM*

By Arthur L. Cohen
Oglethorpe University, Georgia

Introduction. Certain organisms, the Acrasieae, Myxomycetes, and Myxobacteria, lead an undifferentiated existence during the vegetative part of their life cycles, and only at the end of the period of mass increase do they adopt definite reproductive shapes. I am proposing the collective term, opsimorphosis,† to describe this process in which morphogenesis is distinct from and occurs after growth *sensu strictu.*

The Acrasieae are a remarkable group of opsimorphic organisms whose life cycle is as follows: Germination of the spore produces a small ameba, which, for a long time, leads an existence indistinguishable from that of most small soil amebae. Ingestion of bacteria, growth, and multiplication are in typical ameboid fashion. Eventually, the individual amebae culture, and probably also under natural conditions, stream toward a common center for the formation of a fruiting body. This aggregation stage has been called a pseudoplasmodium in analogy with the plasmodium of the Myxomycetes. Each ameba, however, retains its identity even when closely packed by its neighbors. The pseudoplasmodium begins visible differentiation. Some of the amebae become highly vacuolate, secrete firm cell walls and form a parenchymatous stalk on which the other amebae climb or are borne upward as the stalk is continually generated from the center of the mass. The development ends with the sporulation of a mass of cells borne on the tip of the stalk or on the tips of its branches. Each cell invests itself with a spherical to ellipsoidal spore wall and there remains imbedded with its fellows in a mucilaginous droplet until the mass is dispersed.

In the remarkable *Dictyostelium discoideum*, the entire pseudoplasmodium migrates as a slug-shaped mass with a positively phototropic apical papilla before settling down for the final opsimorphic stages. A clear account of the Acrasieae is given by Raper (1940), and of other opsimorphic organisms by Bonner (1952) and Cohen (1948).

For several years, I have been collecting soil samples in an attempt to isolate and culture a wide range of Acrasieae for morphogenetic studies. I found that K. B. Raper was just completing a somewhat parallel project and, with the appearance of his comprehensive review (1951), it would appear that there was little more to be said on the subject. The following account gives methods that extend or differ in one or more respects from those of Raper, and these supplementary notes may serve to encourage even further experimentation with opsimorphic organisms.

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† From the Greek, *opsi*, late; and *morphe*, form.

Collection and preservation. After trials with several different methods of collection, I have developed a routine method of collecting soil samples in 22-ml. vials with plastic screw caps. No attempt is made at sterility, but cross-contamination is guarded against by keeping vials tightly capped before and after use, and by either using the cap to scoop the soil into the vial or by using clean, individually wrapped tongue depressors, which are discarded after use. A collection number on one of the convenient adhesive roll labels (*e.g.*, Dennison *Pres·a·ply Labels*) is affixed to the vial or its cap. These labels do not come off when dampened as do ordinary gummed labels.

Vials should not be filled more than three-quarters full, and may be stored in the refrigerator until used. Soil samples tested, both soon after collecting and after a year's sojourn in the refrigerator, have shown no apparent diminution in variety of opsimorphic organisms. Suspensions kept frozen for months have also exhibited apparently undiminished viability of the different forms.

Culture media. For many years, I have used either dung-decoction agar or sterile dung on nonnutrient agar plates as an enrichment medium with varying results, or else Olive's (1902) method of collecting dung in the field and incubating it in a moist chamber, with very little success. Mannitol agar, as described by Raper (1951 *et prev.*) was somewhat better, but did not give the desired results. A combination of sterile dung and mannitol agar has been found the best medium, so far, and is now used routinely in this laboratory. Autoclaved Ashby's mannitol agar is allowed to cool to approximately 50°C. and is then poured into deep (100 by 15 or 20 mm.) petri plates to a depth of approximately three mm. Firm rabbit dung balls are autoclaved dry, and five are inserted in the agar, one in the center and the other four approximately equidistant from each other and about two cm. from the periphery of the plate. It is important that the dung pellets project above the surface of the agar.

The soil sample is prepared for inoculation by partially filling the vial with water, shaking it vigorously, and then allowing the heavier particles to settle for approximately one minute. The suspension should be turbid but not opaque. Two to three milliliters of the suspension is poured on each plate, which is rapidly tilted to allow as much of the surface as possible to be covered. The plates, after inoculation, are allowed to stand for an hour or two, the excess water then being drained off by carefully tilting them to avoid disturbing spores and cysts which may have settled out. Small pieces of organic debris are left on the plate. They and the dung pellets serve as foci for the fruiting stages of opsimorphic forms.

Moisture relations cannot be too strongly emphasized. Free moisture, aside from a surface film, should have evaporated or have been absorbed within 48 hours, otherwise development of opsimorphic forms is extremely irregular. Plates are incubated at room temperature (20–24°C) and inspected daily under the dissection microscope. Particular attention is paid to the dung balls, the periphery of the plates, and the bits of organic material, for these locations are most likely to be rich in opsimorphic forms.

The Acrasian fructifications when mature, are difficult to distinguish from various fungal sporophores. A suspected sporophore may be examined under the microscope and the parenchymatous stalk is a certain diagnostic feature compared with the linear arrangement of usually elongate cells of true fungal mycelium. With practice, one learns to select the Acrasieae without recourse to high magnification.

Under the conditions stated, within two or three days, there is a copious development of slimy bacteria, apparently mostly *Azotobacter*, followed by Acrasieae within three to ten days simultaneously with the maximal development of mucoraceous fungi. These give way to various penicillia and related forms in about two weeks simultaneously with the appearance of myxobacterial fructifications and myxomycete plasmodia if these appear at all. Later on, *Chaetomium* may cover the dung balls and, in a few instances, *Chondromyces* of the Myxobacteria has made its appearance after six weeks. There is a succession of forms for months, as long as the plates have been kept from drying out.

Occasionally, the maggots of coprophilous flies may be troublesome. Their burrowing and feeding can destroy a plate. The advent of insects may be suppressed by uncovering the freshly inoculated plates and spraying over (not at) them with any of the proprietary aerosol insecticides. A note of caution must be injected here about quarantining these crude plates. They are usually full of mites which can—and have—infested and ruined laboratory cultures.

The isolation medium is a washed suspension of *Escherichia coli* spread on non-nutrient agar (Cohen 1939) buffered at pH 6.0.

Agar (Difco Bacto)	20 gm.
$Na_2HPO_4 \cdot 12H_2O$	0.96
KH_2PO_4	1.45
Distilled water	1 liter

The bacteria are grown at 28° in mass cultures, filtered off (Cohen, 1953b), and washed three times by centrifugation, then suspended in distilled water to give a concentration of 6×10^9 cells/ml. direct count. The concentration is determined nephelometrically. Plates are prepared by first drying the agar slightly in either a desiccator or at 50°C. until 0.6 ml. of the suspension spread over the plate by a rapid horizontal circular motion has its free water nearly absorbed in a half hour. I have found a convenient alternative to standard 100 mm. diameter petri dishes in the small (60 mm. × 15 mm.) petri dishes. If the latter are employed, 5 ml. agar and 0.3 ml. suspension appear to be near optimum.

While routine incubation is at 20°–24°C., temperature does not seem to be as critical a factor with the Acrasieae as with the true Myxomycetes (see Sobels and Cohen, this volume). Plates may be safely stored in the refrigerator without apparent damage to vegetative stages, or may be incubated at a temperature as high as 30°C. Higher temperatures have not been tried but, at 30°, cultures of the Acrasieae grow luxuriantly on *E. coli*. A rapid adaptation to high temperatures may occur in the Acrasieae (Raper, 1951).

For the Acrasieae, monosporangial isolations are made. To make needles fine enough to pick off a single spore mass in a crowded crude culture, soft glass rods are drawn to a diameter of approximately 1 mm., and these shafts are drawn to needles as long and as fine as may be desired. After use, a pair of these needles are simultaneously sterilized and redrawn by fusing the tips together in the Bunsen flame and pulling them apart.

A spore mass is picked up and immediately transferred to the center of a *coli* plate. If the spore mass is wet and the *coli* plate fresh, a good inoculation is certain—the glutinous spore mass clings to the needle and, because of its low surface tension, immediately spreads into the bacteria when the needle is touched to the surface. A drop of water placed on a dry spore mass and in the center of a dry isolation plate will help matters. Successful transfer is first shown by clearing of the bacteria from the center. If the plate is evenly coated with *E. coli*, there is an expanding circle of concentric annuli; vegetative amebae at the periphery, and toward the center, aggregation stages, rising sporangia, and mature sporangia.

I have used several strains of *E. coli* with little apparent difference in their suitability. To facilitate comparisons and aid in the development of uniform culture methods, at present, the strain of *E. coli* employed by Raper is the only one used.

The uniformity of growth is primarily dependent upon the uniformity of the layer of *E. coli*. Comparisons between different strains may be made on this medium with much greater certainty than on media which contain nutrients for the bacteria. In the latter case, the complex metabolic relationships with the consequent changes in the chemical components can have pronounced effects on the morphology of these highly pleomorphic organisms (Cohen, 1953a). For initial studies on the influence of various chemicals on the development of Acrasieae I have made what may be called living two-dimensional chromatograms—rectangular sheets of agar spread with *E. coli*, inoculated along one edge with spores, and with the test reagent applied along the edge at a right angle to the streak of spores.

Routinely, three serial monosporangial transfers are made on isolation plates before final transfer to storage slants.

A dilute (0.5 per cent peptone, 0.5 per cent glucose, 2 per cent agar) nutrient agar is used in this laboratory for maintenance in pure culture. This medium, however, has no advantage over any recommended by Raper, and may be inferior for certain strains to those he lists. It should be stressed* (Raper, 1951) that if one depends on the bacteria present in the slant for inoculation, there is an increasing predominance of slimy forms unsuitable as food for the myxamebae. The bacteria should be inoculated separately from stock tubes at each transfer.

Occurrence in nature. The results from 87 soil samples plated on mannitol-dung medium are given in TABLE 1. The soil samples are from Georgia, Florida, Michigan and Massachusetts. The greatest number of different Acrasieae (four) was obtained from a soil sample collected in a dense pine forest in South Georgia.

The accuracy of this table is limited by the fact that what appeared to be duplicate fructifications were not counted, although probably different strains were represented. Also distinctions between certain habitats were not recognized in early collections, and hence some of them are categorized from memory. Nevertheless, Raper's observations that the Acrasieae are not necessarily coprophilous but are found chiefly on the forest floor is borne out. Much of the bottom land represents low-lying thickets or forest, and the savannahs* are a particular type of forest. (Oosting, 1948).

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* Personal communication from Doctor Ernest Runyon, to whom I am indebted for many stimulating suggestions.

TABLE 1.

FREQUENCY OF ACRASIEAE, MYXOMYCETES, AND MYXOBACTERIA FROM VARIOUS HABITATS AS SHOWN BY DEVELOPMENT ON MANNITOL—RABBIT DUNG AGAR PLATES.

Organism	*Habitat*							
	Forest	Bottom land	Savannah	Pasture	Fields	Sand	Other	Total
Dictyostelium mucoroides	4	6	2	1	0	0	0	13
Dictyostelium giganteum	3	4	0	1	1	0	0	9
Dictyostelium discoideum	2	2	1	0	0	0	0	5
Dictyostelium purpureum	0	0	1	0	0	0	1	2
Dictyostelium "polystelium"	1	2	2	0	0	0	0	5
Polysphondylium pallidum	4	5	1	0	0	0	0	10
Polysphondylium violaceum	3	5	0	0	0	0	0	8
Total Acrasieae	17	24	7	2	1	0	1	52
Myxobacteria	17	14	5	2	4	4	0	46
Myxomycetes	1	5	2	1	0	5	0	14
Total organisms	35	43	14	5	5	9	1	112
Total soils negative	4	5	2	2	1	0	3	17
Total soils	22	26	7	7	5	5	4	87

Horizontally. *Forest:* hardwood or mixed forest, well-drained. *Bottom land:* low-lying wet land bordering streams or marshes, with grasses, shrubs or trees, soil packed, black. *Savannah:* flat more or less open pine—palmetto associations, wet acid floor. *Fields:* cultivated or uncultivated fields or grasslands. *Pasture:* any field used for grazing stock. *Sand:* permanent vegetation-covered coastal dunes or open sandy oak forest with little organic matter.

Vertically. Raper (1951) is probably correct in speaking of *D. mucoroides* and *D. giganteum* as complexes rather than distinct genera. *D. polystelium* is a provisional name given to a new form, branched, shrubby, with some of the characteristics of *Polysphondylium* (see Sussman, this volume). *Myxobacteria* and *Myxomycetes*: the numbers refer to the totals of recognizably distinct forms of Myxobacteria and Myxomycetes respectively found on the individual plates

Summary

Methods are described for the enrichment and isolation of opsimorphic organisms from the soil. The terms *opsimorphic* and *opsimorphosis* are used to characterize those organisms whose morphogenesis is distinct from the vegetative aspects of growth.

A method is given for the uniform culture of Acrasieae to facilitate physiological studies.

An analysis of habitats show most opsimorphic organisms to be inhabitants or forest or rich organic soils.

Acknowledgments

I am deeply indebted to Miss Marianne Falck, my assistant, for her unflagging help; to Doctor Kenneth Raper for suggestions and checking of identifica-

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* The term, *savannah*, is used more or less in the sense of Oosting (1948), though dense pine forests with acid wet soil into which the true open savannah grades is also included here.

tions of the Acrasieae; to Doctor Wallace Alston, President of Agnes Scott College, for his suggestions on transliteration of the Greek combining forms in the newly coined words, *opsimorphosis*, and *opsimorphic*; and to Mr. Alan Dickson, a former student, for soil samples from Michigan.

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THE ISOLATION AND CULTURE OF OPSIMORPHIC ORGANISMS* II. NOTES ON ISOLATION, PURIFICATION, AND MAINTENANCE OF MYXOMYCETE PLASMODIA

By Johanna C. Sobels† and Arthur L. Cohen
Oglethorpe University, Georgia

Introduction. The myxomycetes form a fascinating group of the opsimorphic organisms because of the great variety and range of complexity of the fruiting structures and the fact that, unlike the Acrasieae and Myxobacteria, the vegetative stage (the plasmodium) is physiologically similar to a giant multinucleate ameba (Cohen, 1948). At present, however, they are not nearly as amenable as the Acrasieae to experimental usage, for purification and cultivation are more difficult and, unlike the Acrasieae, which easily repeat their life cycles in the laboratory, the majority of plasmodia do not easily form fruits in the laboratory, nor is there any guarantee that the spores can be germinated or, if germinated, will go on to form a plasmodium.

In order to stimulate an interest in these organisms and to make them more accessible for experimental work, we are presenting our separate and joint experiences on their isolation and cultivation. Further details may be found in previous papers (Cohen 1939, 1941; Sobels 1950) and in the bibliographies of these papers. Detailed descriptions of plasmodia in gross culture are given by Nauss (1943, 1947, 1949) and by Sobels (1948).

Temperature. The optimal temperature for cultivation of the majority of plasmodia is between 20° and 23°C. Prolonged exposure to temperatures higher than 27–30° results in poor growth and eventually in death. In our experience, most plasmodia may be stored for prolonged periods at 16°C. with a consequent and frequently desirable slowing of growth and feeding, while temperatures of more than a degree or two below are harmful. The vegetative plasmodium will not survive freezing. Crude cultures, however, and certainly plasmodia in the field withstand these extreme conditions.

Light. It has been long observed that myxomycete plasmodia are harmed by light, although light appears to be necessary to stimulate fructification (Sobels and Van der Brugge, 1950). Plasmodia should be stored preferably in the dark and should not be exposed to direct sunlight. Many forms can withstand subdued room light successfully.

Moisture. It is a commonplace and incorrect observation that plasmodia are to be found in swampy places. When found (rarely) in swamps, it is our experience that they are inevitably above the water line and, if surface forms, as distinguished from those which live within decaying logs, have full access to the air. They thrive best both in the laboratory and in the field when there is only a film of moisture on the substrate. Some forms (*e.g. Didymium nigripes*, *D. squamulosum*) can live indefinitely under water. The majority,

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† Work done on a Study Grant of The American Association of University Women; a grant of the Phi-Mu Fraternity; and a Fulbright Travel Grant. Present address: Botanical Laboratory, University of Utrecht, Utrecht, Holland.

if covered by water, show the signs of injury described below. It should be noted that they do not appear to be harmed by immersion under inert oils. Plasmodia have been observed under direct oil immersion, a method preferable to using a coverslip.

Concentration of organic metabolites. With the outstanding and almost sole exception of *Fuligo septica*, whose specific name is well-advised, myxomycete plasmodia cannot withstand high concentrations of organic natural substrates. Leaves, decaying wood, soil, *etc.*, are weak media compared to the ordinary laboratory nutrients, and plasmodia quickly die when placed on standard bacteriological media. In crude cultures, it is especially important that the contaminants do not grow luxuriantly or the plasmodium may be overwhelmed and killed. In two-membered culture, certain plasmodia must be watched carefully, especially rapidly growing ones, for they may reach the peak of growth and die overnight.

Reaction of the medium. Myxomycete plasmodia seem to prefer a mildly acid medium—about pH 6.0. Some can be adapted to greater alkalinity by successive transfer on media of gradually increased pH (Cohen, 1939).

Signs of Injury. Response to injury, whether it be caused by light, heat, submergence under water, or chemicals (*i.e.* alkalinity, acidity, metabolites) usually can be easily recognized. The normal vigorous plasmodium has a well-developed fan formed by the anastamosis of the vessels with sheets of protoplasm between the veins which are straight or gradually curved. The absence of a well-defined fan or group of fans, irregular and nodular veins, an open, lax network, and the presence of knobs of protoplasm generally mean unfavorable conditions. Some plasmodia begin to darken from a normally white or pale buff color or, if yellow or orange, turn a deeper hue. The staining of the substrate by pigment indicates a dead or moribund plasmodium.

Adaptions. Plasmodia show striking abilities to adapt to new nutrients (Cohen 1939, 1941; Sobels, 1950). In attempting two-membered or pure culture, it is best to repeat the transfer to the new medium several times, using as large an inoculum as is feasible. Often, growth does not begin until after an induction period which may last for weeks. We have found adaptation to new yeasts and bacteria in two-membered culture, to glycerol as a carbon source, and to autoclaved yeast and yeast autolysate as sole nutrients in pure culture. It is, therefore, unsafe to assume that a plasmodium cannot grow on a new medium if a few transfers fail to develop or even die. It may be mentioned here that, in the experience of one of us (Cohen), the frequently used *Physarum polycephalum* has only its vigorous growth to gigantic size in crude culture to recommend it. Otherwise, it is one of the most variable plasmodia known.

Collection and preliminary purification. As stated previously, cultivation from spores with the exception of a few forms (*Didymium squamulosum*, *Physarum confertum*, *Physarella oblonga*) is a most uncertain procedure. It is best to collect the plasmodia. Collection is more likely to be successful if the plasmodium is protected from drying and from mechanical injury. Plasmodia may be cut with impunity, but crushing appears to result in the release of substances which are harmful even to the mechanically uninjured parts. Leaves and twigs may be placed in jars, folded or bent so that the plasmodium is protected from contact with the walls of the jar or with other objects. Plasmodia on bark, on the under side of logs, and on boards should be carefully cut out with part of the substrate. A sharp penknife is excellent, but the small wood carving sets (*i.e.* the X–ACTO sets) are more versatile. These fragments of the substrate may be protected by bending stiff index cards to act as shields over the plasmodium and securing the shields by rubber bands, thumb tacks, or paper clips. Plastic bags provide good containers. Drying may be avoided by putting a few drops of water on the substrate.

The same protective methods may be used for gathering the delicate fruits of myxomycetes.

When brought into the laboratory, the plasmodia should be placed at once with the underlying substrate on plates of plain 2 per cent agar or on the buffered agar described previously (see Cohen, this volume). The plasmodium will usually—but not always—crawl off the old substrate to the surface of the agar within 24 hours. When the plasmodium has left the old substrate, it should be promptly cut out and with its underlying agar transferred to a new plate. Gross culture may be attempted by sprinkling a few (usually not more than five or six) grains of oatmeal in front of the fan. No more oatmeal should be used than can be covered in less than 24 hours lest molds and other contaminants gain a foothold. A portion of the plasmodium may be transferred to an agar plate on which a washed suspension of yeast, either *Saccharomyces cerevisieae*, *S. ellipsoideus*, or *Torula aclotiana* has been streaked. A thick suspension streaked from a pipette for about 5 cm. across the plate is usually sufficient. If possible, *S. ellipsoideus* or *S. cerevisieae* should be used if the plasmodium grows on it at all, as these yeasts are much more easily grown in mass culture and centrifuged than the slimy *Torulae.*

Rapidly growing plasmodia may be transferred either directly from the initial plain agar plate to oatmeal agar, or preferably from the yeast streaks to oatmeal agar. Although usually impure, a vigorous plasmodium may be able to keep contaminants in check for an indefinite period.

It is preferable, however, to attempt two-membered culture with one of the above yeasts on oatmeal agar in flasks or in large (20–25 mm. diameter) culture tubes. Either 3–4 grains of rolled oats or 0.3 grams are placed in the tube; 10 ml. of 2 per cent agar buffered at pH 6.0 is added and the tube autoclaved. When the tube is cool enough to handle, it is rotated between the palms to suspend the oatmeal and slanted. Some plasmodia do well only on the more concentrated nutrient. At 20–24°C., transfers must be made weekly, or the culture may survive as long as six weeks without transfer, but only experience can be of aid here.

Methods of purification have been described in detail elsewhere (Cohen 1939), but the use of antibiotics (Sobels 1950) can prove a valuable adjunct. Of several antibiotics tested in this laboratory (chloromycetin, terramycin, penicillin G, aureomycin, and streptomycin) only penicillin G and dihydrostreptomycin sulfate were found to be sufficiently non-toxic to the plasmodium for use.

For the following experiment, 200,000 units of crystalline penicillin G and dihydrostreptomycin sulfate equivalent to 1 gm. of dihydrostreptomycin base were dissolved in 100 ml. sterile water. One ml. of this stock solution was added to 9 ml. of either the non-nutrient buffered agar or oatmeal agar in each tube at 50° C., the contents mixed, and the tubes slanted.

Thirteen different plasmodia which had grown in contaminated two-membered culture with *S. cerevisiae* either on oatmeal or on plain agar for 4–5 transfers were inoculated into oatmeal and into *S. cerevisiae* streak tubes containing the antibiotics. Of twelve oatmeal tubes in which transfers survived, five were bacteria-free after one transfer. Of six impure tubes carried into the second transfer, none were freed of the bacteria, indicating that they were contaminated either with bacteria which were initially resistant to these antibiotics or had acquired resistance. The antibiotics had no effect on mold contaminants. Plasmodia are easily rid of molds and yeasts by allowing them to migrate over one or more plates of non-nutrient agar.

Purity is checked by inoculating small portions of a plasmodium into broth tubes of Difco yeast extract, 0.3 per cent and into yeast extract 0.3 per cent plus glucose 1 per cent. It is important that both types of tubes be used, as some microbes require sugar, while others will be suppressed in growth in the yeast-extract-dextrose by the growth of yeast from two-membered cultures or molds from cultures which seem otherwise pure. Peptone should not be used, as it inhibits the growth of some contaminating organisms. Streaking on nutrient plates is not a safe substitute for broth culture. Incubation is at 20–25°C. for at least one week.

Maintenance of cultures. While, in adapting a plasmodium to laboratory culture or to a new medium, it is advantageous to use as large inocula as feasible, for continued cultivation small inocula are preferable. There is a longer period of growth until transfer is again necessary. It must be emphasized that the plasmodial fragment must be cut or broken from the substrate, never scraped or crushed. A convenient tool is a stiff nichrome inoculating needle, the end of which has been flattened into a small sharp-edged spatula. Occasionally, plasmodia crawl off the substrate completely and move to the glass wall of the tube or flask. If portions cannot be lifted from the wall, a small amount of sterile water pipetted against the plasmodium will generally dislodge it and it may be lifted out on a loop or wire hook.

Crude cultures, and sometimes two-membered cultures, may be kept dormant for approximately one year as sclerotia. Sclerotia seldom last longer. In tubes, the plasmodium will crawl toward the dry mouth of the tube and spontaneously sclerotize, or sclerotization may be induced in plates by lifting the cover slightly and allowing the plate to dry gradually.

Finally, the mineral balance must be mentioned. We had found independently that plasmodia could be grown better in some locations than in others, and maintenance in Atlanta was especially difficult. A comparison of our experiences and of the findings of one of us with washed agar (Cohen 1939; Cohen and Sobels, 1952) indicated mineral deficiency, presumably calcium or magnesium since Atlanta water is unusually soft. This was found to be the case, and the present basal medium consists of the buffered agar mentioned previously, but made up with 60 per cent tapwater and 40 per cent of a saturated solution of magnesium and calcium carbonates decanted from the settled suspensions of these salts.

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CELLULAR DIFFERENTIATION IN DICTYOSTELIACEAE: HERITABLE MODIFICATIONS OF THE DEVELOPMENTAL PATTERN*

By Raquel R. Sussman and M. Sussman
Northwestern University, Evanston, Ill.

It is a basic feature of the Protista that the individual cells can be considered units of experimentation. There is, in general, no counterpart to the specialized multicellular organizations encountered in higher forms of life. Despite this and in support of the thesis that discontinuity in nature resides only within the mind of the biologist, one may observe in the Protista tentative approaches to the organized status of metazoans and metaphytes. Thus, many microorganisms grow in a stable if unorganized multicellular mass. Specialized sexual structures are present even in such recognized protists as *Neurospora* and *Achlya*. Finally, at the evolutionary fringes of the group, there exist forms in which the individual cells cooperate during major portions of their life cycle to construct organized multicellular apparatus whose functions contribute substantially to the selective advantage of the species. The slime molds, Dictyosteliaceae, constitute an example of this kind.

Space does not permit a complete description of the investigations of developmental phenomena in these forms. A brief summary of some of the more salient results will be attempted.

Because of the researches of Olive (1902), Raper (1941), Bonner (1944), and others, the life cycles of the Dictyosteliaceae are now well known.

Motile amoeboid cells arise from the germination of thick walled, usually sausage shaped spores. The myxamoebae grow phagotrophically upon a wide variety of bacteria. When the stationary phase has been reached, (under optimal conditions, a total of about 2×10^8 myxamoebae/plate), the cells aggregate to form a multitude of pseudoplasmodia. In two species, the pseudoplasmodia migrate over considerable distances before erecting fruiting bodies. The tips of these plasmodia, representing about one-eighth of the cells, direct the migration and, in their absence, the remaining portions cease to move. Experimentally controlled changes in the direction or position of a tip are followed by reorientation of the entire pseudoplasmodium. The construction of the fruiting body involves a morphogenetic sequence from which emerges a spherical spore mass, stalk, and, in some species, a hold-fast. This basic form is complicated in one genus by the formation of lateral branchings from the main stalk with subsidiary spore masses. Ultimately, the spores are cast off to complete the cycle.

The progeny of single spores or single myxamoebae taken from any stage of the cycle display the normal developmental pattern and produce typical fruiting bodies (Sussman, 1951).

Evidence obtained by Bonner (1947) indicates that the aggregative process is the result of a chemotactic response of outlying cells to a substance termed

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"acrasin" which appears to be produced by cells at or near the centers of aggregation. The same mechanism plays a role in pseudoplasmodial migrations (Bonner, 1949). A study of interspecific and intergeneric cell mixtures (Raper, 1940) showed that not all species can enter into communal aggregates and suggest that the aggregative stimuli are specific.

Two recent publications (Sussman and Nöel, 1952; Sussman, 1952) deal with the populational distribution of the capacity to initiate the formation of aggregative centers. It was found that single cells initiate the centers and that, under the conditions of experiment, only a small proportion of the myxamoebae possess this initiating capacity. For *Dictyostelium discoideum*, the proportion of initiator cells ranged between one per 1000 and one per 2100 cells and appeared to depend somewhat upon the sensitivity of the cells which were responding to the initiating stimulus. For another species, *D. purpureum*, the proportion was estimated at one per 300 myxamoebae.

The later stages of development have been studied extensively (Raper and Fennell, 1952) with particular attention paid to the formation of the stalk and the morphological alterations experienced by the prestalk and prespore cells. The construction of the fruiting body is attended by the extracellular deposition of a polysaccharide, probably cellulosic in nature. The raising of the fruiting structure appears to be the result of the vacuolation and consequent swelling of the stalk cells.

The existence of sexuality in the Dictyosteliaceae has long been suspected as a result of the incidental observations of Olive (1902) and Skupienski (1918). Additional evidence in support of this view has recently been offered (Wilson, 1952) in the form of cytological examination of the myxamoebae during aggregation and pseudoplasmodial migration. The validity of the interpretations drawn by Wilson are, however, contested by Bonner (1952) on the basis of additional cytological data.

At present, no corresponding genetic analysis has been attempted. One reason for this omission has been the absence until recently of a convenient technique for the growth of these forms in clonal isolation. Without such a technique, the phenotypic scoring of suspected segregants could not be accomplished. Equally important, the inability to make single clone isolates precluded attempts at collecting marked stocks for use in a recombination analysis. A previous publication (Sussman, 1951) has eliminated that difficulty. The experimental portion of the present paper will deal with techniques for the procurement of stable variant strains of the Dictyosteliaceae.

All the variants which have been isolated display patterns of cellular differentiation that deviate in one respect or another from the development of the respective wild type stocks. Aside from their potential value as markers in recombination experiments, they represent interesting examples of the extent of genetic control over cellular differentiation. For this reason, detailed descriptions of the variants have been included and their bearing upon problems of development in the slime molds is discussed.

It may be noted that none of the variations has been defined as yet, in biochemical terms. This is due to the fact that the study of the nutritional background and comparative biochemistry of the normally phagotrophic Dictyosteliaceae has progressed only to the point of formulating a partially defined growth medium to replace the bacterial associates (Bradley & Sussman, 1952).

Methods

Organisms and conditions of growth. Four strains comprising three species of slime molds were used: *Dictyostelium discoideum* strain NC-4; *D. discoideum* strain V-12; *D. purpureum* strain V-1; *D. mucoroides* strain S-2. Stocks obtained from single clone isolates were grown in association with *Aerobacter aerogenes* on a glucose-peptone agar medium whose composition has been given previously (Sussman, 1951).

For routine mass transfer passages, a few spore masses were picked with a needle and mixed on a plate with five or six drops of a 48-hour broth culture of bacteria. The mixture was spread with a flamed glass spreader and the plate was incubated at 22°C. Under these conditions a period of 36 to 48 hours is required for the culture to reach the stationary phase. An additional period of up to 24 hours is required for the production of fruiting structures.

When growth in clonal isolation was desired, aliquots of appropriately diluted suspensions were mixed with bacteria and spread. After 3 days at 22°, single spores or vegetative cells give rise to plaques within the layer of bacterial growth. During the fourth day, fruiting structures appear. A plaque count of about 100 per plate represents the upper limit of convenience and accuracy.

Conditions of irradiation. A GE germicidal lamp served as the source of radiation. The cells were deposited on agar surfaces and exposed to the lamp at a distance of 10.5 cm. Either of the following procedures of irradiation and plating were employed.

(1) Spores or vegetative myxamoebae were suspended in Bonner's salt solution (1947). Samples containing $2\text{–}6 \times 10^5$ cells were spread on plates and irradiated for 90 seconds. This dose was found to produce a kill of about 99.99 per cent so that the survivors numbered about 20 to 60 cells/plate. Immediately after irradiation, the plates were respread with a broth culture of bacteria and were incubated. After 4 or 5 days, the plaques which had appeared were examined for evidence of aberrant development.

(2) Plates were seeded with about 10^7 spores or myxamoebae. The interval of exposure was 90 seconds. The survivors numbering about 10^3 cells were respread with bacteria and permitted to grow until the beginning of the stationary phase. At this time, the cells were harvested and replated at a dilution sufficient to provide about 50 plaques per plate. After 4 to 5 days, these plaques were scored for aberrance.

The second method was introduced to recover end point variations which might have occurred several generations after irradiation (Demerec, 1946). Had they not been separated from their wild type sister cells, subsequent to the initial growth, the presence of such variants would have been obscured in the mixed fruitings. The use of the second procedure, however, did not substantially enhance the recovery of variants.

Isolation of the variants. Plaques displaying deviations were picked and plated clonally through several serial passages so that the stability of the novel phenotypes might be examined. Thereafter, slants and lyophilized cultures were prepared. In the case of several stocks which did not form spores, the latter operation had to be omitted.

Results

A. Descriptions of the variants. FIGURES 1 to 5* illustrate the morphological characteristics of some of the variant types. The distinctive features of each variant are summarized below.

(1) Variants of *D. purpureum.*

V-1 aggregateless. Growth is normal but development ceases at the stationary phase, so that aggregation and culmination do not occur. Spores are not produced. Individual differences have been found to exist among the nine independent isolates in this series. The differences concern plaque appearance and the stability of the phenotype. With respect to the latter, the range of stability extends between stocks which have never produced reversions to those so unstable that every plaque contains a few fruiting bodies. Reverted plaques are indistinguishable from the wild type.

V-1 bushy. During the aggregation phase, the pseudoplasmodia become papillated unlike the wild type which forms a smooth rounded slug with a single nipple at the top. When the variant pseudoplasmodia begin culmination, numerous tiny fruiting bodies appear from the papillae giving the whole an appearance of a small bush. Spores are formed and the sori (spore masses) possess the purple pigment of the wild type. No reversions have been observed.

(2) Variants of *D. discoideum*

Nc-4 aggregateless. These stocks are similar to the aggregateless varieties of V-1. The seven independent isolates in this category differ with respect to plaque appearance, stability, and growth rate. A number of them are of particular interest in that the variant amoebae can respond to aggregative stimuli imposed by wild type cells. An analysis of the formation of aggregative centers by such population mixtures has been published elsewhere (Sussman, 1952).

Nc-4 bushy. This variant displays a pattern of development almost identical with that of the V-1 bushy stock. The pseudoplasmodia become papillated and, unlike the wild type, do not migrate. Numerous tiny fruiting bodies appear in clusters and produce the usual yellow pigment of this species. Spores are formed. An appreciable fraction of the fruitings remain in intermediate stages of maturation. No reversion to wild type has been observed but spontaneous transformation to aggregateless varieties is frequent.

Nc-4 fruitless-1. This variety grows and aggregates in normal fashion. At that stage, the development ceases. Ultimately the cells are gathered in small regular hemispherical mounds. Some spore formation occurs.

V-12 fruitless-1. This type is indistinguishable from the previous stock

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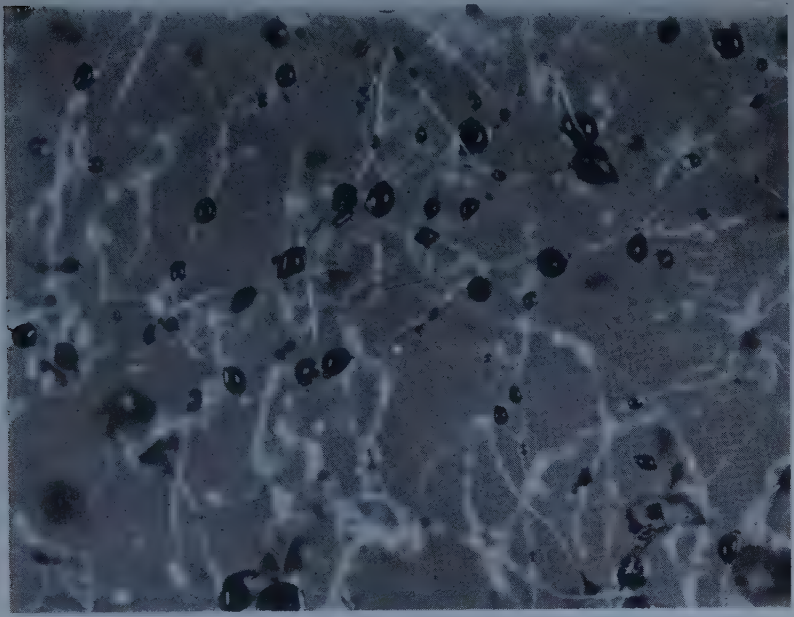
* Photographs taken at 7.5× magnification.



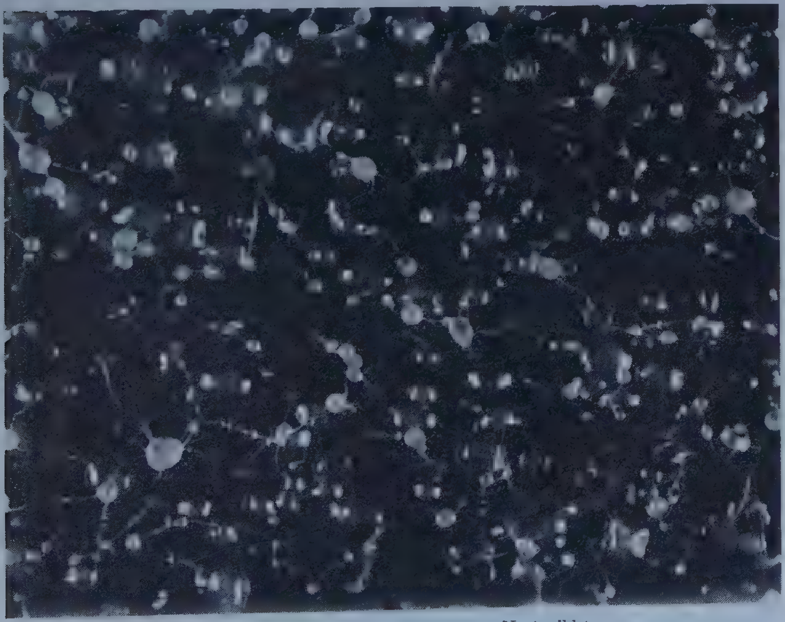
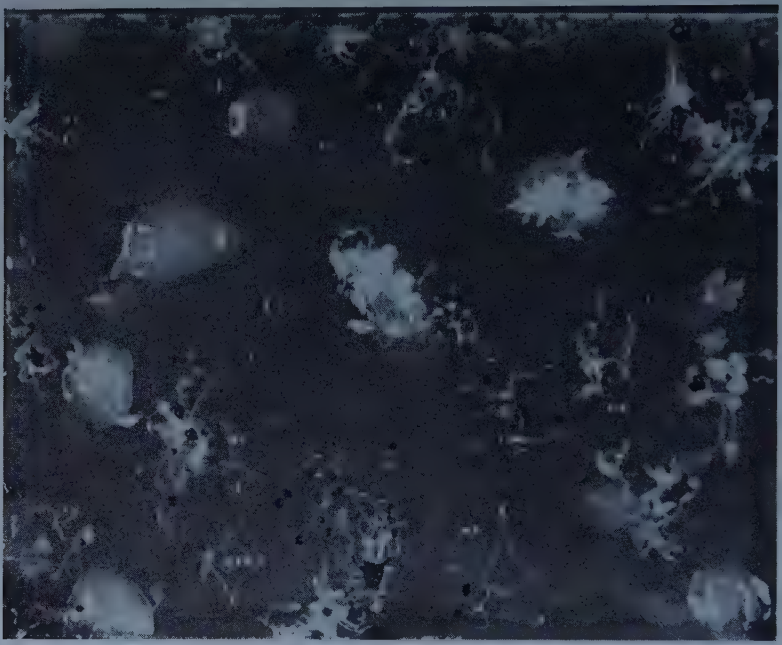


Figure 1. Upper: V-1 wild type. Lower: Nc-4 wild type.



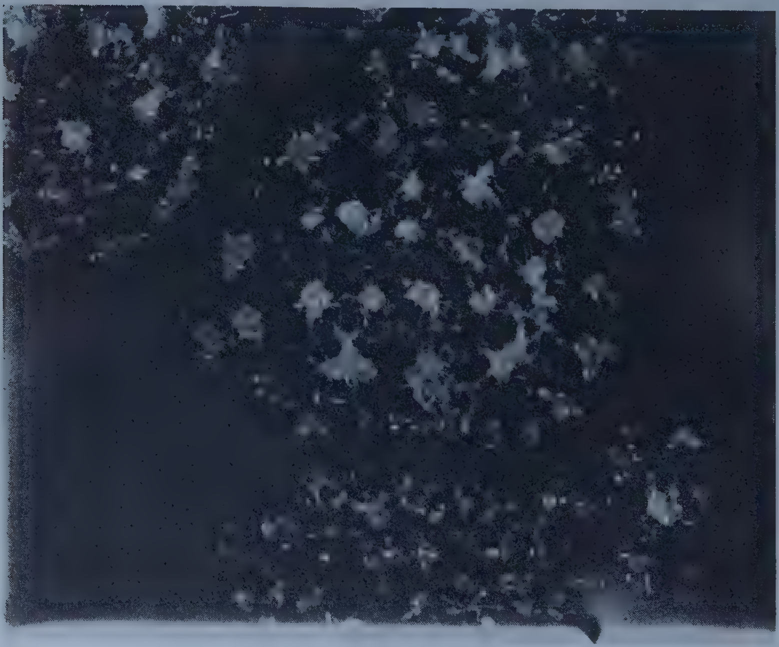
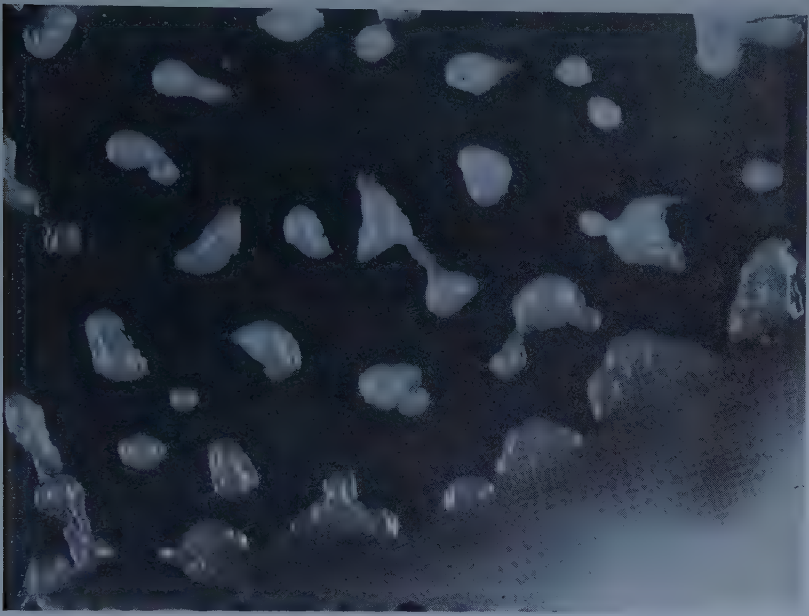


FIGURE 2. Upper: V-1 bushy. Lower: Nc-4 bushy.



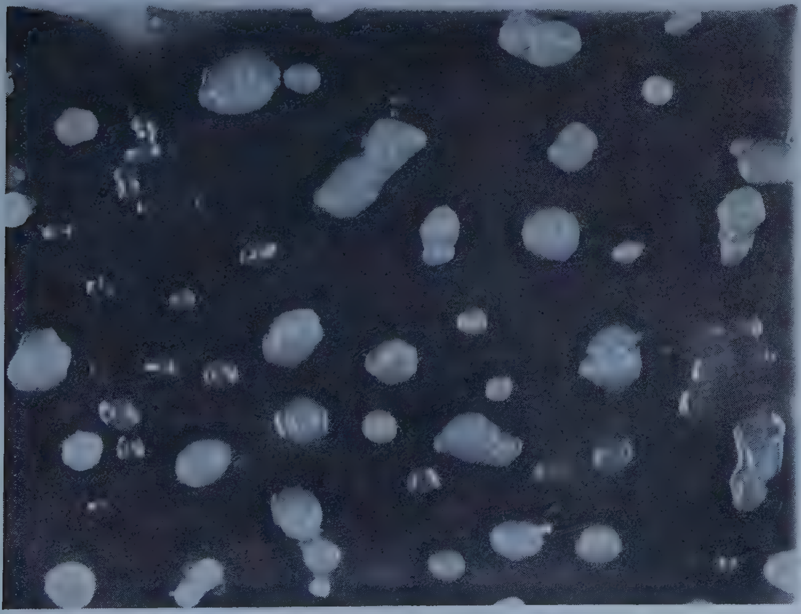


Figure 3. Upper: Nc-4 fruitless-1. Lower: S-2 fruitless-1.

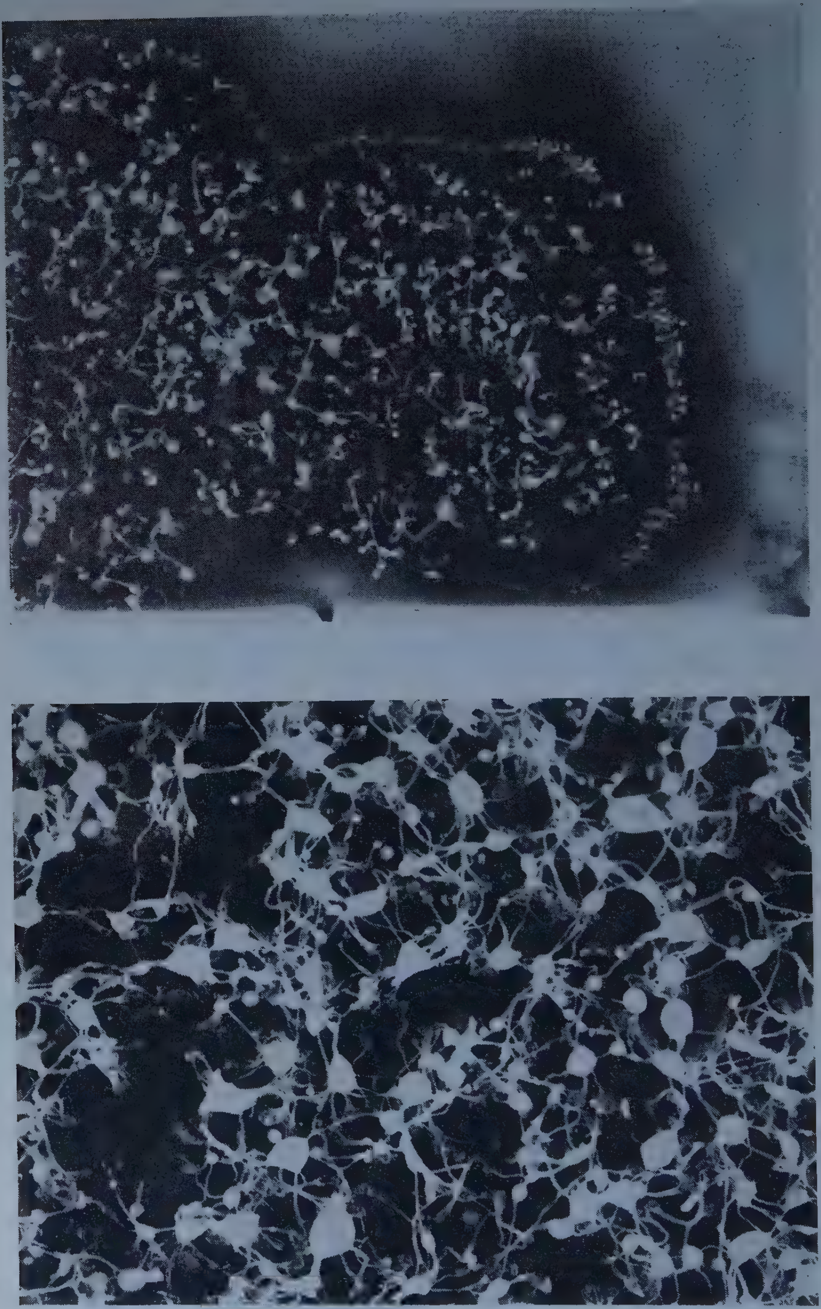


Figure 4. Upper: Nc-4 dwarf. Lower: S-2 wild type.

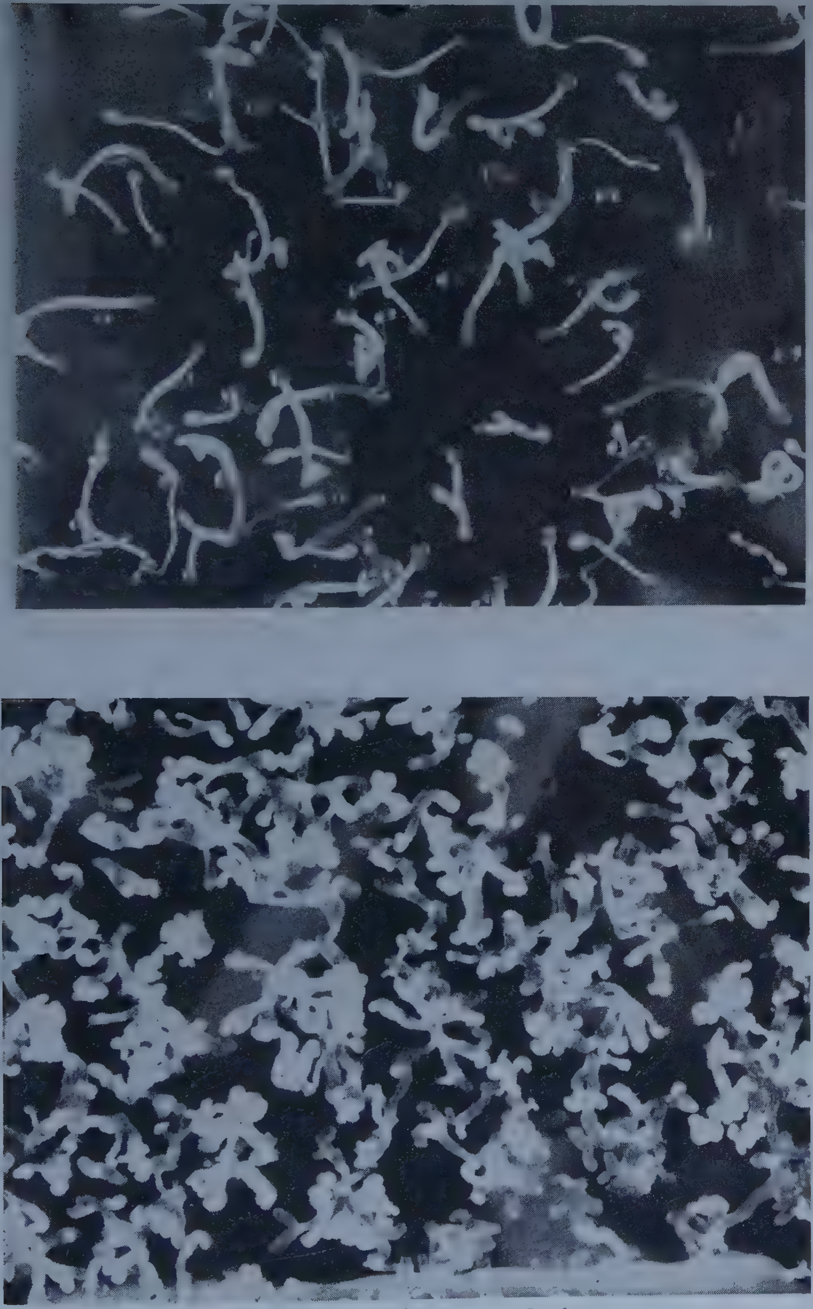


FIGURE 5. Upper: S-2 forked. Lower: S-2 glassy.

except that the texture of the mounds is perhaps a bit more mucoid. However this is also a characteristic of V-12 wild type.

V-12 fruitless-2. Normal development proceeds in this strain through aggregation but stops at intermediate stages in the erection of the fruiting bodies. The degree of completion varies between the typical mound of the fruitless-1 type and almost mature fruitings.

Nc-4 dwarf. The developmental cycle is normal except for the fact that the fruiting bodies are extremely small. The different components are proportionately reduced in size so that the structures are normal but miniature. No reversions have been observed.

V-12 dwarf. This stock is indistinguishable from the previous one.

(3) Variants of *D. mucoroides*

S-2 aggregateless. This stock is almost identical to the aggregateless variants of the other species.

S-2 fruitless-1. The appearance of the strain is wholly like that of the fruitless-1 variants of *D. discoideum* except for the fact that the mounds are a little more regular.

TABLE 1.

Variant type	Parent strain	No. plaques examined	No. isolates	No. variants/ plaque examined
Aggregateless	Nc-4	5100	7	1.4×10^{-3}
	V-1	6900	9	1.3×10^{-3}
Bushy	Nc-4	5100	1	2×10^{-4}
	V-1	6900	1	1.5×10^{-4}
Fruitless-1	Nc-4	4800	1	2.1×10^{-4}

The approximate frequencies of appearance of some variant types among irradiated myxamoebae.

S-2 fruitless-2. Like the previous strain regular mounds of cells appear but, at the apices of these mounds, one generally finds long thin stems and occasionally a mature sorus. The stock is extremely pleomorphic in this respect.

S-2 forked. Development is normal up to culmination. At this point, the process deviates to the extent that sorocarps with very thick straight stalks are produced in contrast to the wild type whose stems are very thin and crinkled. Usually, very small twin sori branch off diagonally from the top of the stem. The thickness and slimy quality of the stalk suggest an excessive production of slime sheath material. This variant is extremely stable and no back mutations have been observed. Subsequent fruitless or aggregateless variants derived from this stock always revert to the forked type and never to wild type.

S-2 glassy. Again the development is normal up to culmination. The fruiting bodies are extremely thick with no demarcation between stalk and sorus although spores are produced. The fruitings are so intertwined and curled that they appear to branch from the same stems. The crystalline appearance and thickness of the stems produce an effect of extreme rigidity. No reversions have been observed.

B. The frequency of occurrence of the variations. Table 1 contains data which illustrate the frequency with which some of these variations arise in irradiated populations. We consider the figures to be grossly inaccurate and at best to serve only as approximations.

Discussion

Although the number of variants thus far isolated is relatively small, a spectrum of aberrance has emerged which suggests the existence of two general patterns of developmental variation in the Dictyosteliaceae. One such category is exemplified by the stocks known as aggregateless, fruitless-1 and fruitless-2. In these forms, the usual developmental process appears to have halted at intermediate stages. The situation would seem to be much like that encountered in biosynthetically deficient mutants and indeed may actually constitute the morphogenetic expression of enzymatic deficiencies. The second pattern is exemplified by the stocks known as bushy, forked, glassy, etc. In these forms, the developmental process does not appear to have halted but rather to have been conducted along deviant pathways. The identification of these aberrancies with enzymological abnormalities should help to elucidate the normal course of cellular differentiation displayed by the slime molds.

Another consideration which may prove to be of significant import lies in the fact that the different species and strains can give rise to very similar variants. For example, the bushy character has arisen in Nc-4 and V-1; the fruitless-1 in Nc-4, V-12, S-2, and V-1; the fruitless-2 in V-12 and S-2; the aggregateless in Nc-4, S-2 and V-1. This suggests, albeit not compellingly so, that the nature of genetic control over the cellular differentiation may be common to all of the species examined and that the metabolic activities which lead to the production of heterogeneity may be similar as well.

The question of whether or not the variant stocks represent genic mutations cannot be settled at this time. To arrive at a decisive answer, one must have access to a means of segregation analysis. Since, however, a major object of this investigation has been to isolate aberrant strains precisely for the purpose of employing them as markers in a demonstration of genetic segregation, we are in the unfortunate predicament of having to prove what we think exists by what we hope occurs.

Summary

Techniques have been described for the isolation of variant strains of *Dictyosteliaceae.*

The variants which have been isolated display patterns of development that deviate from those of the wild type species. The bearing of these variations upon the problem of genetic control over cellular differentiation in the slime molds is discussed.

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UTILIZATION OF PURINES FOR NUCLEIC ACID SYNTHESIS IN CHRYSOMONADS AND OTHER ORGANISMS*

By Leonard Hamilton†
Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, New York, N.Y.

The discovery by Stock *et al.*[1, 2, 3] that 2,6-diaminopurine inhibited kappa in *Paramecium aurelia* led to a study by us of the incorporation of C^{14} labeled purines into the nucleic acids of *P. aurelia*. In view of the different patterns of purine incorporation established in *Paramecium*[4, 5] and that described by Flavin and Graff for *Tetrahymena geleii*,[6, 7] it was of interest to determine the purine utilization pattern for nucleic acid synthesis of another very different protozoan. Accordingly the utilization of purines has been studied in a chrysomonad flagellate available in culture in synthetic media.

The results indicate that chrysomonads, like all species which have been studied, utilize adenine for the synthesis of polynucleotide adenine and, like all but *Tetrahymena geleii* and *Lactobacillus leichmannii*, transform exogenous adenine into polynucleotide guanine. In contrast to rat and mouse but in common with other species, chrysomonads readily utilize guanine for polynucleotide guanine; similarly, like all species except the rat, the mouse, and yeast, they readily transform exogenous guanine into polynucleotide adenine. Similar but not identical results obtain with 2,6-diaminopurine, a purine not yet found in nature. The present communication presents these results and reviews the patterns of purine incorporation in different species.

Experimental

The chrysomonad was *Ochromonas malhamensis* (Pringsheim strain), obtained from Doctor S. H. Hutner of the Haskins Laboratories and maintained as described by Hutner *et al.*[8] For the incorporation studies, the chrysomonads were inoculated into 500 ml. lots of synthetic medium (TABLE 1). This represents an earlier version of the synthetic medium.[op cit.] Individual experiments were conducted in which 5 mg. adenine-8-C^{14} with activity of 16,200 cpm/μmol., 5 mg. guanine-8-C^{14} with activity of 21,900 cpm/μmol., and 4.9 mg. 2,6-diaminopurine-2-C^{14} with activity of 13,100 cpm/μmol.,‡ each dissolved in 10 ml. of water and previously sterilized by autoclaving for 20 minutes at 118–120°C., were added to different lots of medium.

The chrysomonads were incubated for 168 hours under "warm-white" fluorescent lamps, at between 28 to 30°C. The chrysomonads were spun down by centrifugation, washed with alcohol and extracted three times with ice-cold 5 per cent (w/v) trichloroacetic acid to remove adhering free purines and acid-soluble nucleosides and nucleotides. The material was then defatted and dried by 3 extractions with a hot alcohol-ether (3:1) solution and finally washed

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with ether and air-dried. In the experiment in which adenine had been added to the medium, 310 mg. of defatted chrysomonads were obtained. In those in which guanine and 2,6-diaminopurine were used, the corresponding yields were 232 mg. and 205 mg. respectively.

The purines were isolated from the polynucleotides of the defatted material and their activity determined by methods detailed elsewhere.[9] In brief, the pentose nucleic acid (PNA) and desoxyribonucleic acid (DNA) were separated by alkaline hydrolysis of the PNA, followed by acid precipitation of DNA. The PNA nucleotides and DNA were further hydrolysed with H_2SO_4 and the resulting purines, obtained as silver salts, were liberated with HCl. The HCl was removed by repeated evaporations to dryness *in vacuo*. The purines were then dissolved in measured quantities of dilute HCl and aliquots were separated *via* paper chromatography. The adenine and guanine spots were eluted with HCl and the amount of purine was determined spectrophotometrically. An aliquot of the eluate was evaporated to dryness to remove HCl, then transferred to an aluminum planchet and the activity measured in an internal Geiger-Müller flow counter (Tracer Laboratories SC 16, helium-isobutane mixture). The samples were counted to within 3 per cent error. The purity of the isolated purines was determined by paper chromatograms and by the ratios of the optical densities of the eluted purines at wavelengths 250, 260, 280, and 290 mμ.

TABLE 1
CHRYSOMONAD MEDIUM

pH 4.9–5.4

	mg./100 ml.		mg./100 ml.
$CaCO_3$	15.0	Mo	2.5
L-glutamic acid	200	L-Histidine HCl	50.0
Na H glutamate	200	L-Arginine HCl	50.0
$(NH_4)_2H$ Citrate	80.0	Sucrose	1000
K_2HPO_4	30.0	Thiamine	0.2
$MgSO_4 \cdot 7H_2O$	20.0	Biotin	0.5 μg./100 ml
DL-Methionine	40.0	Cystine	2.0
Metals Mixture	1 ml.		

Metals Mixture 1 ml

EDTA (free acid) 5.0 mg.; Mn 2.0 mg.; Zn 2.5 mg.; Fe 0.25 mg.; Co 0.06 mg.; Cu 0.01 mg.; Boron 0.02 mg.; Iodine 0.001 mg.; pH approx. 3.5.

Results

The findings, summarized in TABLE 2, show that the chrysomonads are able to utilize adenine for the synthesis of polynucleotide PNA-adenine and for the transformation of adenine into polynucleotide guanine. Analysis of the DNA purines indicates that the chrysomonads are likewise able to utilize adenine for the synthesis of polynucleotide DNA-adenine and guanine. The DNA-adenine had an activity of 3,330 counts/min./μmol. and the corresponding value for the DNA-guanine was 1,600 counts/min./μmol. It is of interest that the ratio of radioactivity in adenine to that of guanine is approximately the same (2:1) in both PNA and DNA. Similarly, chrysomonads utilize guanine and 2,6-diaminopurine for polynucleotide PNA-guanine and for the transformation of guanine and 2,6-diaminopurine into polynucleotide adenine. In the utilization of guanine, the ratio of radioactivity in guanine to that of adenine is approximately 3:1 and the corresponding value for 2,6-diaminopurine is 2:1. In the experiments with guanine and 2,6-diaminopurine, the desoxyribonucleic acid purines were obtained in amounts too small to give satisfactory results.

TABLE 2

INCORPORATION OF C^{14}-LABELED PURINES INTO PNA OF CHRYSOMONAD *Ochromonas malhamensis* (PRINGSHEIM STRAIN)

Offered in medium				Recovered in PNA						
				Adenine			Guanine			Adenine and Guanine* total counts
C^{14}-Purine	μM	Counts* per μM	Total* counts	μM	Counts* per μM	Total* counts	μM	Counts* per μM	Total* counts	
Adenine	37	16,200	599,000	9.5	6460	61,400	7.3	2,900	21,400	82,800
Guanine	33.3	21,900	729,000	8.6	5250	45,200	6.7	18,400	123,000	168,000
2,6-diamino-purine	32.4	13,100	426,000	6.3	500	3,150	5.8	1,290	7,500	10,600

* Radioactivity in counts/min.

TABLE 3

INCORPORATION OF C^{14}-LABELED PURINES INTO PNA OF CHRYSOMONAD *Ochromonas malhamensis* (PRINGSHEIM STRAIN); A COMPARISON OF DATA WITH DIFFERENT CHROMATOGRAPHIC MEDIA AND ON DIFFERENT COUNTERS

C^{14} Purine	Purine Isolated	BuOH-NH$_4$OH		IsopropOH-HCl	
		μM × 10^{-2}*	Counts† per μM	μM × 10^{-2}*	Counts† per μM
Adenine	Adenine	15.2	6,460	5.2	4,840
	Guanine	11.7	2,930	5.5	2,060
Guanine	Adenine	13.8	5,250	15.4	5,500
	Guanine	10.8	18,400	15.6	17,950
2,6-Diaminopurine	Adenine	10.2	500	7.6	690
	Guanine	9.3	1,290	6.2	1,370

* Actual quantity of purine eluted from paper chromatogram.
† Radioactivity in counts/min./μmol.

In contrast to the extensive utilization of adenine and guanine, and especially the latter, by chrysomonads, the utilization of 2,6-diaminopurine is comparatively meager. Thus, on a molar basis, 84 per cent of PNA-guanine and 24 per cent of PNA-adenine were derived from the exogenous labeled guanine, whereas the chrysomonad synthesized only 10 per cent of PNA-guanine and 4 per cent of PNA-adenine from exogeneous 2,6-diaminopurine. Judged by the total counts recovered in PNA-adenine and guanine compared with the total added to the medium, this difference in the utilization of guanine and 2,6-diaminopurine by the chrysomonads appears even greater. Thus, the chrysomonad utilized 17 per cent and 6 per cent respectively of the available guanine for PNA-guanine and adenine respectively. The corresponding values with 2,6-diaminopurine were 2 and 0.7 per cent respectively.

In view of the possibility that amounts of guanine larger than 10 micrograms may not have been recovered quantitatively under the neutral conditions of the *n*-butanol-aqueous ammonia solvent system,[10] the purine samples, which had been stored in solution for approximately one year at 4°C. were again separated by a mixture of isopropanol-HCl.[11] The activity of the isolated purines was then determined on another counter (Radiation Counter Laboratories Counter Mark 13, Model 3, helium-isobutane mixture). The results are summarized in TABLE 3. It is of interest that in this solvent system the molar relationships of PNA-adenine and guanine approximate more nearly to unity. The differences in the activity observed in the experiment in which adenine was used may be due to the small amounts of purines that were isolated from the stored solution and also in part to the use of a different counter.

Discussion

The diversity in patterns of purine utilization for polynucleotide synthesis by various organisms lends interest to the intermediate position of the chrysomonads between *Tetrahymena* and the rat. The rat at one end of the spectrum of species studied utilizes adenine for polynucleotide adenine synthesis and and transforms it into polynucleotide guanine, but hardly utilizes guanine; guanine or 2,6-diaminopurine are not transformed into polynucleotide adenine. *T. geleii* and *L. leichmannii* at the opposite end utilize adenine for polynucleotide adenine but do not transform it into polynucleotide guanine; whereas they utilize guanine for polynucleotide guanine and transform it into polynucleotide adenine.

In early experiments with isotopes, Plentl and Schoenheimer[12] fed guanine labeled with N^{15} in the 1 to 3 positions both to rats and pigeons and could detect no significant amount of the isotope in the nucleic acids isolated from the tissues although labeled allantoin was excreted by the rat and labeled uric acid by the pigeon. Brown and co-workers[13] by the use of similarly labeled adenine showed that the Sherman strain white rat utilized it for polynucleotide adenine and transformed it into polynucleotide guanine. With N^{15}-labeled guanine they were also unable to detect any utilization. These findings on the utilization of adenine in the Sherman rat have been extensively confirmed following the feeding or intraperitoneal injection of C^{14}-labeled adenine; exogenous adenine is utilized for PNA- and DNA-adenine and guanine in a variety of tissues that have been examined including liver, small intestine, large intestine and stomach.[14, 15] In addition, by the use of C^{14}-labeled guanine injected intraperitoneally in Sherman rats, it has been possible to detect a very low incorporation of guanine into PNA-guanine but no transformation into polynucleotide adenine.[16] The dilution factor of 1,000 obtained in polynucleotide guanine following the administration of C^{14} guanine is to be compared with dilution factors of 8 to 20 observed following adenine administration under comparable conditions. In contrast the Sherman rat uses 2,6-diaminopurine with relatively low dilutions for the synthesis of PNA- and DNA-guanine but not for adenine.[18]

In contrast with the intact rat, Abrams has reported that erythroid bone marrow slices of the rabbit, previously made hyperplastic by treatment with a hemolytic agent, phenylhydrazine, utilize adenine and guanine for both PNA- and DNA-adenine and guanine synthesis and in addition transform a small quantity of adenine into polynucleotide guanine and vice versa.[17]

In the C57 black mouse, it has been found that dietary N^{15} adenine is incorporated into both the polynucleotide adenine and guanine but that this occurs to a much smaller extent than in the Sherman rat.[16] There is a very small incorporation of N^{15}-guanine into polynucleotide guanine.[19] Similarly, 2,6-diaminopurine is utilized for polynucleotide guanine.[20] The present data are not adequate to indicate whether the mouse does or does not accomplish the reverse transformation of guanine into polynucleotide adenine.

The utilization of adenine in yeast for the synthesis of both PNA-adenine and guanine parallels the findings in the rat, although there is more extensive transformation of adenine into guanine with the yeast.[21] Unlike the rat and the mouse, growing yeast readily utilizes guanine for PNA-guanine but the labeled guanine is not transformed into polynucleotide adenine to an appreciable extent. As in the mouse, the data are not adequate to indicate definitely whether guanine is or is not transformed into polynucleotide adenine. It is of interest that in yeast the transformation of exogenous adenine into polynucleotide guanine is inhibited in the presence of guanine in amounts sufficient for PNA-guanine synthesis.[21] This indicates that the presence of an unlabeled purine in a nutrient medium can influence the apparent transformation of labeled purine for polynucleotide synthesis. This possible factor must be borne in mind in the interpretation of comparative patterns of purine utilization.

Aerobacter aerogenes, the bacterium on which *P. aurelia* is fed, represents a stage further in this pattern of purine metabolism in that not only can it utilize adenine for PNA-adenine and transform this with almost equal efficiency into PNA-guanine, but it can also readily utilize guanine and 2,6-diaminopurine for PNA- and DNA-guanine and transform small but quite significant quantities of both purines into polynucleotide adenine.[4, 5, 9] This facility to utilize guanine and/or 2,6-diaminopurine for polynucleotide adenine appears relative stronger in *P. aurelia*, in experiments in which living *A. aerogenes* are also present. *P. aurelia*, variety 4, stock 51 killer, mating type VII (51-7K) utilizes adenine equally well for PNA-adenine and guanine; in the identical organism without kappa, the "sensitive" paramecium, the radioactivity of the PNA-adenine is 2 to 1.5 times as great as that in the PNA-guanine. Both varieties utilize 2,6-diaminopurine for PNA-guanine and transform a relatively appreciable proportion into PNA-adenine.

The chrysomonads most nearly resemble the *Paramecium* in their utilization of exogenous purines but they differ in the extent to which they make use of guanine as compared with 2,6-diaminopurine for polynucleotide synthesis. In this, they almost present the reverse pattern of the Sherman rat which, on a lesser scale, hardly utilizes guanine at all for polynucleotide guanine whereas it makes much more efficient use of 2,6-diaminopurine.

In *Escherichia coli*, the conversions of one purine to the other is dependent on the purine concentration in the medium.[22] Adenine at concentrations of

2.6 μm. per liter is utilized equally well for PNA- and DNA-adenine and guanine and at concentrations below this it is used preferentially for polynucleotide adenine. The conversion of guanine to polynucleotide adenine is not as complete as that of adenine into polynucleotide guanine and here again there is the same dependence of utilization on the concentration in the medium. These transformations of the labeled purines in *E. coli* appear to be largely independent of the presence of the other labeled purine in the medium.

There seems to be a tendency among the different species for the increasing facility to transform adenine into polynucleotide guanine to be paralleled by a comparably increased facility in transforming guanine into polynucleotide adenine. This extent of interconversion is maximal in the versatile *L. casei* which, in this respect, occupies a point midway between the rat and *T. geleii*. It utilizes adenine and guanine equally efficiently for both PNA-adenine and PNA-guanine. Unlike yeast, and *L. leichmannii*, the interconversions are not influenced by other purines in the medium.[23] *L. casei* also utilizes 2,6-diaminopurine extensively as a precurser of both PNA- and DNA-adenine and guanine; utilization and transformation of diaminopurine is equal at low concentrations but at inhibitory concentrations of diaminopurine there is preferential utilization for polynucleotide guanine.[24]

T. geleii and *L. leichmannii* exhibit a pattern in purine utilization almost the complete reverse of that shown by the rat. In *Tetrahymena*, guanine serves equally well as a precursor of polynucleotide guanine and adenine[6] and adenine appears to be utilized only for polynucleotide adenine.[7] In these experiments guanine was supplied at a concentration just less than that which would have permitted half-maximal growth. This raises the possibility that this unlabeled guanine may have exerted a sparing action masking any transformation of adenine into polynucleotide guanine. The low specific activity of the adenine used would also hinder detection of a small amount of such a transformation. The present information on *T. geleii* does not, therefore, exclude the possibility that exogenous adenine can be transformed into polynucleotide guanine to a small extent.

L. leichmannii has a pattern similar to *T. geleii*. It utilizes guanine-8-C^{14} and guanine-2-C^{14} equally well for polynucleotide guanine and adenine but utilizes adenine only for polynucleotide adenine.[25, 26] Contrasting with the behavior of yeast in which guanine spares the transformation of exogenous adenine into polynucleotide guanine, in *L. leichmannii* adenine spares the transformation of guanine into polynucleotide adenine; the incorporation into polynucleotide guanine remains unaffected.

Most organisms do not require pre-formed purines for nucleic acid synthesis. Utilization of exogenous purine for most species represents a by-passing of a portion of the usual synthetic pathways. The differences in utilization of adenine, guanine, and 2,6-diaminopurine for polynucleotide adenine and guanine suggest that the preformed purines enter the synthesis of nucleic acids at a stage later than that of a common purine precursor. Differences in purine utilization and transformation suggest differences in the accessibility and capacity of the metabolic pathways from the common purine precursor to adenylic and guanylic acids and possibly between each other.

Summary

The chrysomonad *Ochromonas malhamensis* (Pringsheim strain) utilizes adenine for the synthesis of polynucleotide adenine and transforms it into polynucleotide guanine. Guanine and 2,6-diaminopurine are likewise utilized for polynucleotide guanine and to a lesser extent transformed into polynucleotide adenine. This pattern of purine utilization has been compared with those of the rat, the mouse, yeast, *A. aerogenes*, *P. aurelia*, *E. coli*, *L. casei*, *T. geleii, and L. leichmannii.*

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DISCUSSION OF PAPERS ON CILIATE NUTRITION

By W. H. Johnson
Wabash College, Crawfordsville, Indiana

I have been much interested in the growth requirements of *Paramecium* ever since I first obtained growth of *P. multimicronucleatum* in 1941 using filter-sterilized pressed yeast juice.[1] For about three years, I carried cultures of this organism in a proteose peptone-pressed yeast juice medium with a fission rate of about one every 48 hours. In 1945, Tatum and I reported that there are two factors in the p.y.j. necessary for growth of *Paramecium*, one heat labile and one heat stable. The heat stable factor can be removed by dialysis and the heat labile factor can be precipitated from the whole p.y.j. at ¾ saturation with ammonium sulphate.[2]

Numerous attempts were made to substitute known substances for these two fractions of p.y.j. In January of this year, I published results of work in which I had found it possible to substitute hydrolyzed nucleic acid, and then later, cytidylic and guanylic acids, for the heat labile factor. This made it possible to eliminate the tedious process of filter-sterilizing the yeast juice. The medium used after that was an autoclaved medium of proteose peptone, the fluid part of autoclave p.y.j., and cytidylic and guanylic acids. Prior to this, in 1949, van Wagtendonk and Hackett had described an autoclaved medium for *P. aurelia*. This consisted of autoclaved yeast autolysate (Basamin Busch) and a lettuce infusion in which *Aerobacter aerogenes* had grown for 24 hours and then autoclaved. I obtained some of this medium from Doctor van Wagtendonk and found that *P. multimicronucleatum* would grow successfully in it. I also furnished Doctor van Wagtendonk and his group some of my medium and they found that *P. aurelia* could be cultured in it. So the requirements of the two species are similar in many respects.

I have tried in many ways to substitute known substances for both the proteose peptone and the yeast in my medium. Early this year, Mr. Miller of Doctor van Wagtendonk's group, furnished me some of their lemon juice preparation. I have tried this in the culture of *P. multimicronucleatum*, simply adding it to the regular stock medium, and have found it to be definitely stimulatory. After three days of culture, there are approximately twice as many organisms in the cultures containing the lemon fraction as in the control cultures. Attempts were made to determine some of the specific requirements of *P. multimicronucleatum* by using the medium described by van Wagtendonk, Miller and Conner in the Federation Proceedings for March 1952 which includes salts, vitamins, dialyzed yeast, dialyzed proteose peptone, and the lemon fraction.[3] At first, it appeared that cultures could be maintained in this medium. However, I have been unable to carry them past the fourth transfer.

In the work reported by Doctor van Wagtendonk, undialyzed proteose peptone was used. Apparently, in the dialysis, something in the proteose peptone is lost which is essential. In this connection, I should like to ask Doctor van Wagtendonk: "How many transfers have you been able to carry *P. aurelia* through in the present study using undialyzed proteose peptone?"

DOCTOR VAN WAGTENDONK: "We have carried the animals through approximately 100 4-day transfers in our present medium."

The nature of this lemon factor, so similar to cholesterol in structure, is most interesting. We have tried cholesterol in our work in a wide range of concentrations and it has always appeared to have a definitely harmful effect, as judged by the length of life of the organisms in the cultures containing it.

In addition to the lemon factor, I am interested in the yeast preparation used by the Indiana group. In my work, up until I started using the yeast preparation described by Doctor van Wagtendonk today, I had always used p.y.j. I have tried a variety of commercial yeast preparations without results. Yet this yeast preparation gives good results. In using the yeast of the Indiana workers, thriving stock cultures of *P. multimicronucleatum* can be maintained by adding only this yeast preparation to the proteose peptone medium. It is not necessary to add cytidylic and guanylic acids as it was when the autoclaved p.y.j. was used. In this connection I would like to ask Doctor van Wagtendonk: "How have you determined whether cytidylic and guanylic acids are necessary in your present medium?"

DOCTOR VAN WAGTENDONK: "Guanylic and cytidylic acids have been routinely included in the medium, because Doctor Johnson had found them essential for the growth of *P. multimicronucleatum.* We have not yet shown that these two acids are essential for *P. aurelia.*"

Also I want to ask: "Has the group at Indiana tried Kidder's medium for *Tetrahymena* in their experiments?"

DOCTOR VAN WAGTENDONK: "We have tried Kidder's medium without success. First of all, we found the Tweens definitely toxic to *P. aurelia.* When the Tweens are omitted no growth takes place at all, even with the addition of the factor from lemon juice. We are investigating the proteose peptone and the yeast extract fraction and hope to be able to replace these two with known compounds."

We have made numerous attempts to use it in our work. It now seems clear that the use of the Tweens is harmful to *P. multimicronucleatum.* When Kidder's medium is used with the Tweens omitted, the organisms live for a considerable period of time and undergo a few divisions, but successful transfers have not been possible. We have had difficulty in obtaining a clear medium using the Kidder formula. Some of the salts precipitate upon autoclaving. Recently, we have found that the use of EDTA prevents this and gives a clear medium. In preparing this, we have autoclaved the salts with EDTA and water and then have added aseptically the other ingredients. Paramecia in this medium live for some time and show some divisions. It may be that this medium will merit further use as the other unknown factors are determined.

At present, it would appear that there is a substance (or substances) in the proteose peptone essential for the growth of *Paramecium* and also a necessary substance in the dialyzed yeast fraction. In connection with this essential material in the yeast fraction, the work of Lilly is quite interesting. It would seem that the substance in yeast which Lilly has found necessary for the culture of carniverous hypotrichs feeding on *Tetrahymena* may be the same unknown substance in the yeast which is necessary for the growth of *Paramecium.* Doctor Lilly has said that he would send me a sample of this material when he has enough available and I am anxious to test it with *Paramecium.*

*References**

1. Johnson, W. H. & E. G. S. Baker 1942. Science **95:** 333.
2. Johnson, W. H. & E. L. Tatum 1945. Physiol. Zoöl. **8:** 163.
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* References not included in Doctor van Wagtendonk's paper.

NUTRITION OF *HERPETOMONAS* (*STRIGOMONAS*) *CULICIDARUM*

By Jean Cowperthwaite, Morton M. Weber,[*] L. Packer,[†] and S. H. Hutner
Haskins Laboratories, New York, N.Y.

Introduction

No group offers more diverse and numerous species for the study of the comparative biochemical evolution of parasitism than does the Trypanosomidae. Unfortunately, little biochemical study of this family has been possible because of the difficulty encountered in cultivation and maintenance of many species.[23, 26] Indeed, among the pathogens some have been impossible to culture *in vitro* (*e.g.*, *Trypanosoma equiperdum*, *Trypanosoma congolense*) and, in others, where cultures have been obtained, infectivity has not always been retained.[23] In view of the limited success in identifying growth factors for exacting members of the Trypansomidae,[15] it was thought that other, non-pathogenic parasites found in this family, and possessing relatively simple growth requirements, would lend themselves more easily to nutritional studies. It was assumed that certain physiological characteristics would be common to both pathogenic and non-pathogenic groups, and that consequently a study of a representative of the latter group would be a stepping-stone to the identification of requirements for the more fastidious groups.

The blood requirement of many trypanosomes and Leishmanias has been difficult to satisfy with simpler materials. Certain parasites grow in the presence of hemin plus serum as a blood substitute (*Leishmania agamae*, *Leishmania ceramodactyli*) but many serum requirements still defy analysis[15] (*cf.* Sprince[20]). *Herpetomonas culicidarum*, a mosquito parasite in the family Trypanosomidae, has, however, a blood requirement that can be completely replaced by hemin.[15] The remaining growth factors are thermostable and are found in ordinary peptones. These considerations plus the ease of handling and lack of pathogenicity made us suppose that the nutritional requirements of this parasite could be identified by procedures successful with many exacting protists. Its morphological relationship to *Leishmania* and to *Trypanosoma cruzi* indicated that its nutritional requirements might be similar in the main to both these pathogenic groups.

Herpetomonas culcidarum[‡] was first isolated by Noguchi and Tilden[16] in 1926 by streaking a saline suspension of infected mosquito gut on blood agar. M. Lwoff demonstrated in 1940,[15] with the aid of hydrolyzed silk, that thiamine was essential. Little work has been done since.

Materials. Experimental media were contained in 25-ml. flasks incubated at 25–28°C. The total volume in each flask was 5 ml. The flasks were covered with glass or aluminum caps. When tubes were used instead of flasks, they permitted poor or inconsistent growth. The reason for this has not been determined. It was probably not lack of oxygen, as no increase in rate or amount of growth was noticed on either a reciprocal or a slow rotary shaker.

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[*] Present address: Department of Microbiology, The Johns Hopkins University, School of Hygiene and Public Health, Baltimore, Maryland.

[†] Present address: Department of Microbiology, Yale University, New Haven, Connecticut.

[‡] Nomenclature in this group is badly confused. Possible synonyms found in the literature for this organism are numerous. To mention a few: *Herpetomonas culicis*, *Leptomonas fasciculata*, *Leptomonas michiganensis*, *Crithidia anophelis*. *Strigomonas culicidarum*, *Strigomonas fasciculata*, etc. As several able workers have recently addressed themselves to the task of unravelling this situation and, as we have not carried out morphological studies, the designation of the organism in this paper is only for convenience at this time and follows that used by Noguchi and Tilden.

Solutions. Experimental basal media were made up in double or quadruple strength for handling and storage. Quadruple-strength bases did not precipitate if the energy source was omitted and then added just before use. The vitamin supplement was kept in a separate solution and added to the base before autoclaving. Unstable vitamins (*e.g.*, folic acid and citrovorum factor) were stored in solutions of 100 μg./ml. of 20 per cent ethanol which had been made slightly alkaline to dissolve the vitamin. The solutions were then immediately neutralized and stored in the freezing compartment of a refrigerator[10] until just before use. All other solutions were kept with volatile preservative[8] in the refrigerator. Experiments were performed by adding various nutrients to the concentrated base and diluting to final volume. Media were autoclaved for 30 minutes at 118–121°C.

Inoculum. The inoculum was prepared by centrifuging a stock culture and resuspending in sterile appropriately deficient basal medium. This washing was repeated and the organisms were suspended in the original volume of basal medium, then finally diluted 4× with sterile deficient basal medium. Experimental flasks were inoculated with one drop of this suspension. Use of a washed stock culture for inoculating did not always result in low blanks. When vitamin curves were desired an inoculum grown in the "first" synthetic medium shown in TABLE 1 was used with 0.3 ml. of 15-unit liver (Lederle) per 100 ml. of final base added. In this case, mere resuspension of the flagellates in deficient medium and dilution was adequate to eliminate any carry-over effects.

The flasks were incubated for 7 to 14 days. Growth was measured in optical density units.

Experimental

In replacing peptone, a change to completely synthetic media was not at first attempted. Instead, use was made of fairly well-defined materials such as acid hydrolyzed gelatin* as an amino acid source, hydrolyzed yeast nucleic acid† as a purine and pyrimidine source, and a complete mixture of known thermostable vitamins (shown in TABLE 1). Energy source (glucose added aseptically, or sorbitol added before autoclaving—both at 1 per cent levels), salts (as shown in TABLE 1), and hemin were added. This base supported growth only if a liver preparation† was present.

Methods

Maintenance. A strain of *Herpetomonas culicidarum* originally isolated by Noguchi from *Anopheles* was obtained from Doctor A. Lwoff. It was maintained on the following stock medium at room temperature (22–25°C.):

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* 500 g. of Eastman Kodak "Pract."gelatin were suspended in 2000 ml. of 10 per cent (by weight) H_2SO_4. The mixture was autoclaved 8 hours at 115-120°C., then brought to pH 2.5 with powdered barium hydroxide. Twenty-five g. of norit was added and the precipitate was filtered off with the aid of "Filter Cel". The filtrate was brought to pH 4.0 with additional barium hydroxide, and to pH 6.8 with NaOH. After boiling to expel CO_2, the pH was finally brought to 7.0 with NaOH and the solution filtered. The filtrate was made to 2500 ml. to make a 20 per cent solution.

† 5 g. of commercial yeast ribonucleic acid was suspended in 50 ml. of H_2O. NaOH was added dropwise until all was dissolved and the final pH was 9.0. The solution was autoclaved 5 minutes at 115–120°C., then brought to pH 7.0 with HCl and made to 125 ml.

Yeast autolysate (Albimi)	1.0 g.
Sorbitol	1.5 g.
"15 unit" liver concentrate	0.1 ml.
K_3 citrate·H_2O	0.02 g.
Triethanolamine*	0.5 g.
Hemin*	2.5 mg.

pH 8.1
H_2O to make 100 ml.

TABLE 1

COMPOSITION OF "FIRST"[a] AND "FINAL"[b] SYNTHETIC MEDIUM FOR *Herpetomonas culicidarum*
Amounts in 100 ml. of final base

KH_2PO_4	0.02 g.	Sorbitol	1.5 g.
Ethylenediamine-tetra-acetic acid	0.06 g.	Triethanolamine	0.5 g.
$MgSO_4 \cdot 7H_2O$	0.04 g.	Adenine	1.0 mg.
Fe (as $SO_4^{=}$)	0.1 mg.	Guanylic acid*	1.0 mg.
Ca(as Cl^-)	0.2 mg.	Hemin (see p. 975)	2.5 mg.
NaCl*	0.05 g.	$(NH_4)_2HPO_4$*	0.008 g.
Metal mix No. 32†	2.5 ml.	pH 8.1	

AMINO ACID MIX		*VITAMIN MIX*	
L-Arginine HCl	25 mg.	Thiamine·HCl	0.6 mg.
DL-Aspartic acid*	50 mg.	Pyridoxamine·2HCl	0.1 mg.
L-Cystine*	1 mg.	Riboflavin[b]	0.04 mg.
L-Glutamic acid*	100 mg.	Ca pantothenate	0.3 mg.
Glycine*	50 mg.	Nicotinic acid	0.4 mg.
L-Histidine·HCl·H_2O	20 mg.	Folic acid[b]	25.0 μg.
DL-Isoleucine	5 mg.	Biotin	1.0 μg.
DL-Leucine	5 mg.	B_{12}*	0.1 μg.
DL-Lysine	20 mg.	Citrovorum factor* ††	0.2 μg.
DL-Methionine	6 mg.	Putrescine·2HCl*	0.04 mg.
DL-Phenylalanine	4 mg.	*p*-Aminobenzoic acid*	0.01 mg.
DL-Tryptophan	2 mg.	Choline*	1.0 mg.
DL-Threonine	10 mg.	Inositol*	1.0 mg.
L-Tyrosine	5 mg.	Thymine*	1.0 mg.
DL-Valine	6 mg.	Orotic acid*	0.3 mg.
		Protogen*	200 units

[a] For growth in "first" synthetic medium add 15-unit liver 0.01 ml. to 0.05 ml. per 100 ml. of final medium.
[b] In final synthetic medium: riboflavin 0.1 mg. and folic acid 100 μg.
* Compounds found unnecessary for growth in "final" synthetic medium.
† Metal mix: $ZnSO_4 \cdot 7H_2O$, 4.4 g., $MnSO_4 \cdot H_2O$, 3.08 g., $Na_2MoO_4 \cdot 2H_2O$, 1.01 g., $CoSO_4 \cdot 7H_2O$, 95 mg., $CuSO_4 \cdot 5H_2O$, 39 mg., H_3BO_3, 28.4 mg., NaI, 1.2 mg., H_2O to make one liter. Completely dissolve Na molybdate first, then add others, completely dissolving each before adding the next.
†† Supplied as "Leucovorin" (Lederle) as the Ca·$5H_2O$ salt.

Subcultures were made once a month and incubated at room temperature. Well-grown cultures could be kept at 6°C. for many months without loss of viability.

By supplying liver extract at a minimal level it was possible to demonstrate requirements for many vitamins and amino acids by stepwise substitution of various combinations of amino acids for the gelatin hydrolysate, and purines and pyrimidines for hydrolyzed yeast nucleic acid. The introduction of vitamins and other essentials by liver extract was a serious difficulty and was obviated in several ways:

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* The triethanolamine was supplied from a 50 per cent (w/v) aqueous solution. Before the triethanolamine was added to the medium, it was used to dissolve the hemin. This medium was tubed and autoclaved.

(1) Fortunately, several kinds of liver preparations* could be used. These varied in their relative content of likely essentials. Thus, a riboflavin requirement could be detected when "Liver L" was used and not with other liver preparations. Many amino acid requirements could be detected in the presence of "1:20" liver for which a "15-unit" liver preparation was unsuitable.

(2) Treatment of the various liver fractions at pH 2 or 10 for 20 minutes at 121°c. destroyed many known essentials. Requirements for pyridoxamine, thiamine, riboflavin, and folic acid could be detected in this manner.

(3) Photo-inactivation of liver fractions was also tried as a means of identifying a vitamin B_{12} requirement. A riboflavin requirement could be confirmed by this means but no B_{12} requirement could be detected. This was corroborated in later experiments with synthetic media.

Results

After many experiments of the type just described, and others defining such variables as pH, buffer, and substrate (some details are given under the subsequent discussion of the medium), most of the peptone portion of the medium was replaced with the "first" synthetic medium given in TABLE 1 to which a smaller amount of liver fraction (0.01 ml. of 15-unit liver per 100 ml. of final base) had to be added.

A. *Discussion of medium in* TABLE 1.

Hemin. Hemin is highly insoluble in water but soluble in alkaline solutions. As it is known that hemin polymerizes in alkaline solution, thus probably becoming partly unavailable to the organism, compounds forming soluble complexes with hemin (*e.g.*, caffeine and histidine) were assayed. These were found to have no sparing effect on the amount of hemin required. Finally, it was found that the buffer, triethanolamine, was an excellent solvent for hemin. Hemin was dissolved in 50 per cent (w/v) aqueous triethanolamine to make a solution containing 5 mg./ml. This solution was stable indefinitely at room temperature. When the required amount of hemin was added to the base from this solution, only half the required amount of triethanolamine had been added. The other half of the triethanolamine was added to the medium before the addition of triethanolamine-hemin mixture as the medium should not be very acid when hemin is added.

Not all commercial samples of hemin were suitable. One sample which had been recrystallized by the manufacturer was found to be completely inactive. This may have been due to polymerization which can occur during recrystallization procedures.[12] Consistently satisfactory lots were obtained from the Nutritional Biochemicals Corporation.

pH. The medium was adjusted to a high pH value (8.1) for several reasons. Sugars were excellent substrates but consequent production of acid had to be controlled not only to prevent killing of the organisms but to prevent hemin precipitation (a condition resulting in cessation of any further growth). Hemin precipitates at about pH 7.4.[14] A pH up to 8.4 was well tolerated.

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* Either "15-unit liver" (Lederle), Wilson's "Liver L" (an enzymatic digest of the 70 per cent alcohol-insoluble portion of liver), or "Liver 1:20" (an aqueous extract of liver) could be used as growth supplement.

Triethanolamine. This buffer was used because the range of solubility of hemin in culture media fell within its buffering range (pK_a 7.77). Its solubility and lack of toxicity allowed use of high concentrations. Until buffers of this type were used, heavy growth could not be obtained in partly defined media.

Energy. Sorbitol was supplied as a thermostable substrate, an important consideration when autoclaving at a high pH. Glucose was used occasionally as a substrate and was added aseptically. It gave more rapid but not heavier final growth.

Vitamin mix and amino acid mix. Some of these requirements could not be definitely established or eliminated because of the masking effects of the necessary liver fraction. Therefore, a complete amino acid and vitamin supplement was used to avoid possible deficiencies of known nutrients. To simplify the task of replacing the liver fraction, all the thermostable metabolites were used which have thus far been shown to be required by other heterotrophic organisms. Most vitamins were supplied in excess.

B. *Replacement of liver fraction.*

It was noticed that a doubling of the amount of the vitamin mix in TABLE 1 added to this basal medium had a sparing effect on the liver fraction needed. It was thought that the vitamins, although already present in excess as judged from the reports on other microorganisms, might nevertheless be too low. Consequently, the concentration of all vitamins was tripled and this resulted finally in growth without any liver fraction. The effective vitamins were folic acid and riboflavin, each at high levels sparing the other at still higher levels. The values required for this interaction are shown in TABLE 2. Although thymine also spared folic acid, no combination of thymine, riboflavin, and folic acid brought their effective concentrations down to the physiological level.

Folic acid is virtually absent from liver concentrates[19, 24] and, in particular, from the sample of "15-unit" liver used by us.* Citrovorum factor is present in 15 unit liver but *Herpetomonas culicidarum* did not respond to citrovorum factor under these conditions. From these considerations it was concluded that the actual metabolite in natural materials had not yet been identified.

C. *Simplification of medium and identification of known requirements.*

With the elimination of liver, the absolute requirements could now be determined and the medium simplified. The simplified "final" synthetic medium is shown in TABLE 1. The amino acids and vitamins listed are absolute requirements (with the possible exception of DL-threonine which may be required under certain conditions, as yet not fully defined). Curves for folic acid, nicotinic acid, riboflavin, biotin, and pantothenate showed that generally high levels of these were required. Curves for thiamine and pyridoxamine were not determined. All subsequent experiments were done with this medium.

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* We are indebted to Doctor H. P. Broquist of the Lederle Laboratories for assays of folic acid in this preparation: the *Streptococcus faecalis* method indicated a content of 1.0 μg./ml.

D. *Sparing of folic acid requirement.*

In attempting to spare the high folic acid requirement several possibilities were considered:

(1) The right form of folic had not been supplied.

(2) The organism might be using a breakdown product of folic acid.

TABLE 2

INTERACTIONS OF FOLIC ACID, THYMINE, AND RIBOFLAVIN

	No thymine					Thymine 0.5 mg. %					Thymine 2.0 mg. %				
	Riboflavin mg. %					Riboflavin mg. %					Riboflavin mg. %				
	0	0.02	0.04	0.08	0.2	0	0.02	0.04	0.08	0.2	0	0.02	0.04	0.08	0.2
No folic	0	0.01*	0.02	0.02	0.02	0	0.01	0.02	0.01	0.02	0	0.01	0.01	0.01	0.01
Folic acid 1.0 μg.%	0	0.02	0.01	0.01	0.02	0	0.01	0.02	0.02	0.02	0	0.02	0.02	0.01	0.02
Folic acid 3.0 μg.%	0	0.02	0.02	0.02	0.02	0	0.02	0.02	0.02	0.04	0	0.02	0.02	0.02	0.02
Folic acid 10.0 μg.%	0	0.02	0.03	0.03	0.04	0	0.03	0.05	0.04	0.05	0	0.03	0.05	0.05	0.06
Folic acid 25.0 μg.%	0	0.08	0.06	0.06	0.38	0	0.04	0.11	0.71	0.75	0	0.52	1.12	1.12	1.30
Folic acid 50.0 μg.%	0	0.12	0.11	0.20	0.38	0	0.21	0.41	1.12	1.30	0	1.04	1.30	1.24	1.24

Basal medium used was "first" synthetic base (TABLE 1). Folic acid, riboflavin, thymine, and citrovorum factor were omitted.

* Growth is in terms of optical density.

TABLE 3

FOLIC-SPARING ACTIVITY OF NUCLEOSIDES

	folic acid μg. %			
	10	25	35	50
No addition	0.06	0.06	—	—
Adenine 1.0 mg.%	0.26	0.76	1.16	1.20
Guanine 1.0 mg.%	0.16	0.48	—	—
Adenosine 0.1 mg.%	0.14	0.16	—	—
" 0.5 mg.%	0.68	0.58	—	—
" 1.0 mg.%	0.90	1.04	—	—
" 3.0 mg.%	1.20	0.98	—	—
Guanosine 0.5 mg.%	0.60	0.54	—	—
" 1.0 mg.%	0.80	0.78	—	—
" 3.0 mg.%	1.24	1.34	—	—

Medium: "final" synthetic medium (TABLE 1). Adenine and folic acid were omitted.

(3) The synthesis of one or more compounds in which folic acid is involved might be inefficient, requiring very high concentrations of this vitamin.

(1) *Forms of folic acid.* Citrovorum factor, a compound closely related in structure to folic acid, showing greater activity than folic acid for some organisms,[3, 4] was almost completely inactive in this system. Analogs of folic acid were tried, such as aminopterin (4-amino folic acid) and 10-formyl-folic acid. Both of these were active at approximately the same level as folic acid itself. These two compounds are usually potent antagonists of folic acid in both animal and bacterial metabolism.[17] Aminopterin in high concentration has also been shown to have folic acid activity for *Tetrahymena geleii*.[9, 22]

(2) *Breakdown products of folic acid.* Pteroic acid was active but not more so than folic acid. Other parts of the folic acid molecule were inactive (TABLE 4, column I, and TABLE 5, first column) such as xanthopterin, *p*-aminobenzoic acid (PABA), xanthopterin + PABA, *p*-aminobenzoylglutamate, and 2-amino-4-hydroxy-6-carboxypteridine.

TABLE 4

COMPARISON OF ADENOSINE AND D-RIBOSE FOR FOLIC-SPARING ACTIVITY

		I	II	III
No addition		0.04	0.01	0.02
Folic acid	0.5 μg.%	0.03	0.02	0.96
	2.0 μg.%	0.05	0.02	0.82
	10.0 μg.%	0.06	0.08	1.12
	50.0 μg.%	1.08	1.12	0.90
Pteroic acid	0.05 μg.%	0.04	0.03	0.98
	2.0 μg.%	0.04	0.02	0.92
	10.0 μg.%	1.00	0.14	0.94
	50.0 μg.%	1.12	1.08	0.94
Citrovorum factor	100.0 μg.%	0.05	0.04	1.20
	200.0 μg.%	0.05	0.05	0.90
	500.0 μg.%	0.11	0.06	1.12

I = adenine 1 mg. per cent
II = adenine 1 mg. per cent + D-ribose 3 mg. per cent
III = adenosine 5 mg. per cent
Basal medium as shown in TABLE 1 ("final" synthetic) with folic acid and adenine omitted.

TABLE 5

EFFECT OF ADENOSINE ON THE ACTIVITY OF PARTS OF FOLIC ACID MOLECULE AND OF CITROVORUM FACTOR

			I
No addition		0	0
Folic acid	0.1 μg.%	0.02	1.00
	1.0 μg.%	0	1.24
	10.0 μg.%	0.06	1.20
	100.0 μg.%	0.94	1.20
PABA	10.0 μg.%	0	0
	30.0 μg.%	0.01	0
Xanthopterin	1.0 μg.%	0	0
	10.0 μg.%	0	0
	100.0 μg.%	0	0
Citrovorum factor	0.1 μg.%	0	0.98
	1.0 μg.%	0	1.00
	10.0 μg.%	0.01	1.20
	100.0 μg.%	0.04	1.30
	500.0 μg.%	1.12	1.24

I = adenosine 5 mg. per cent
Basal medium as shown in TABLE 1 ("final" synthetic) with folic acid omitted.

(3) *Inability to synthesize certain compounds.* Folic acid is involved in the synthesis of serine and glycine,[1, 25] and in the formation of the purine and pyrimidine ring.[25] The role of folic acid in the metabolism of formate and in the synthesis of these compounds was considered.[18, 25] None of these com-

pounds, singly or in combination, showed any sparing of the folic acid requirement. When nucleic acid derivatives were tried, nucleosides were found to have remarkable activity in sparing folic acid. Adenosine and guanosine were equally active (TABLE 3). Free guanine or adenine was completely inactive in this respect. There was, however, an absolute requirement for purines independent of the folic acid requirement. The only nucleotide tested, adenosine-3-phosphoric acid, was inactive in satisfying the nucleoside requirement. A combination of D-ribose and purines did not replace ribosides (TABLE 4).

E. *The folic acid requirement in the presence of nucleosides.*

The magnitude and specificity of the folic acid requirement was redetermined in the presence of adenosine (TABLES 4 and 5). Folic acid now gave a response at physiological levels, citrovorum factor was as active as folic acid, and most of the previously tried breakdown products remained inactive. Pteroic acid still replaced folic acid in the presence of adenosine, and was also active at lower levels, although a few experiments show that it may not be quite as active as folic acid or citrovorum factor under these conditions.

Discussion

Herpetomonas culicidarum is the first member of the Trypanosomidae to be cultured in synthetic media. One important obstacle was that of solubilizing hemin; this was overcome by the use of efficient soluble buffers and alkaline media. Alkaline media, in turn, would have been useless without solubilizing metal-binders, as precipitates of salts otherwise make these media unusable.

The vitamin and amino acid requirements of *Herpetomonas culicidarum* resemble those of other fastidious bacteria and protozoa. The level of vitamins required by *Herpetomonas* was in many instances higher, but this might be a reflection of the non-physiological composition of the basal medium (*e.g.*, many of the amino acids were racemic) or it may be that adenosine is still not the active material supplied from natural sources such as liver.

A specific nucleoside requirement is unusual. Many organisms can use purines, nucleosides, and nucleotides interchangeably. A few require nucleosides or nucleotides and cannot use free purines or pyrimidines.[6, 7, 13] An unusual situation was reported by Gunsalus *et al.*[5] who found that among the requirements for the oxidation of pyruvate by cell suspensions of *Streptococcus faecalis* was a ribonucleoside. The nucleoside could not be replaced by purines, and substitution of nucleotides caused a considerable decrease in activity. Since in this case ribose was found to be the active portion of the nucleoside molecule, this specificity is not comparable to that of *Herpetomonas*.

The lack of activity of nucleotides may simply be explained by a lack of phosphatase activity in *Herpetomonas culicidarum*, rendering it unable to convert nucleotides into the corresponding nucleosides. Then if *Herpetomonas* uses nucleosides for nucleic acid synthesis (a hypothesis which is supported by the close relationship of the adenosine requirement to folic acid), the synthesis apparently does not proceed from nucleoside to nucleotide as is usually assumed.

Other functions of adenosine for *Herpetomonas culicidarum* cannot be ruled out since in a formal sense adenosine is part of several coenzymes. The fact that guanosine is also active further supports the idea that exogenous nucleosides are not incorporated directly in coenzymes but that their main function in this flagellate is in nucleic acid synthesis.

Whether the active material in liver extracts, as assayed in the presence of physiological levels of added folic acid (15-unit liver has a limited content of folic acid or citrovorum factor), is adenosine or a combination of nucleosides remains to be determined. Rough calculations indicate that adenosine is unlikely to account for all the activity of liver extracts. On a weight basis (1 ml. = 100 mg. dry weight), the amount of liver found necessary to replace adenosine was 1.0 mg. per cent. Adenosine itself is active only at levels of 1.0 mg. per cent and sometimes more is required. This would imply that if ribosides were the active material in this liver concentrate, all of the concentrate would have to be riboside.

The nature of the active material in liver and the general distribution of this activity in nature are subjects awaiting investigation. Whether this activity depends in part on hitherto unrecognized forms of folic acid (*e.g.*, that described by Swenseid *et al.*[21] and by Lascelles and Woods[11]) and nucleosides also remains to be determined. It is important to identify the active materials in liver, blood, and hemopoietic tissue, because such information should help in developing synthetic media for those blood parasites which either cannot now be grown on simplified media or have not been grown in artificial culture at all.

Summary and Conclusions

A synthetic medium of a degree of complexity comparable to that required for exacting bacteria such as *Streptococcus* or *Lactobacillus* was developed for the insect parasite, *Herpetomonas culicidarum.*

The quantitative vitamin requirements of this parasite are high as compared with corresponding requirements in other microorganisms.

Extremely high concentrations of folic acid, required for growth in this synthetic medium, could be spared by adenosine or certain other nucleosides. Adenylic acid was ineffective in this respect, as were also purines, pyrimidines, and ribose.

Citrovorum factor was almost completely inactive in substituting for folic acid in the absence of adenosine, but exhibited full activity in the presence of adenosine.

The probable presence of another active factor in liver, different from nucleosides, is discussed.

Acknowledgments

We are indebted to Doctor H. P. Broquist and his co-workers of the Lederle Laboratories Division of the American Cyanamid Company for many stimulating discussions, for the folic acid derivatives and citrovorum factor, and for the occasional use of his laboratory facilities by one of us (J. C.). This work was assisted by grants from the Lederle Laboratories and the Rockefeller Foundation.

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THE ENZYME SYSTEMS OF THE CULTURE FORM OF *TRYPANOSOMA CRUZI*

By Harry D. Baernstein
Federal Security Agency, Public Health Service, National Institutes of Health, National Microbiological Institute, Bethesda, Maryland*

A study of the enzyme systems of *Trypanosoma cruzi* offers an opportunity to contribute to comparative biochemistry, to a rational chemotherapy of Chagas's disease, and to the cultivation of *Endamoeba histolytica* (Phillips, 1950; Phillips and Rees, 1950; Phillips, 1951).

Previous investigations of *T. cruzi* grown *in vitro* indicate that this flagellate consumes oxygen and produces carbon dioxide. Cyanide and azide inhibit oxygen consumption indicating heavy metal catalysis (von Brand, Johnson, and Rees, 1946). Hemin and ascorbic acid are required in the medium (Lwoff, 1938). Glucose and fructose are utilized but not maltose nor lactose. Succinate, pyruvate, lactate, and formate were found in the medium after harvesting (Chang, 1948).

This paper presents a summary of our work over the last two years on this organism. Some of the results have already been published; some are in press; and some are presented for the first time.

Materials and Methods

The Culbertson strain of *T. cruzi* was grown in the liquid medium of Phillips (1951), harvested, washed, and homogenized. Activities are expressed in terms of total nitrogen. Details of these methods may be found in the earlier papers.

Results

It is well known that *Trypanosoma cruzi* utilizes glucose. This utilization is probably preceded by phosphorylation although the enzymes concerned have not yet been characterized. We have shown that hexose diphosphate is split into trioses by an aldolase found in homogenates of this organism (Baernstein and Rees, 1952).

(1) *Hexose diphosphate* → *3-phosphoglyceraldehyde + dihydroxyacetone phosphate.*

FIGURE 1 shows that the rate of triose production from hexose diphosphate as measured by the increase in 2,4-dinitrophenylhydrazones, expressed in terms of alkali-labile phosphate (Sibley and Lehninger, 1949), is linear for at least 40 minutes when 0.236 mg. trypanosome nitrogen reacts with 0.005 M hexose diphosphate in the presence of 0.056 M hydrazine at pH 7.5. For further characterization of this enzyme, the original paper must be consulted.

The presence of triose phosphate isomerase was shown indirectly by determining the effect of the fixative, hydrazine, on the recovery of triose by the hydrazone method of Sibley and Lehninger (1949). The interpretation was confirmed by substituting crystalline aldolase, free from isomerase* for the homogenate (TABLE 1).

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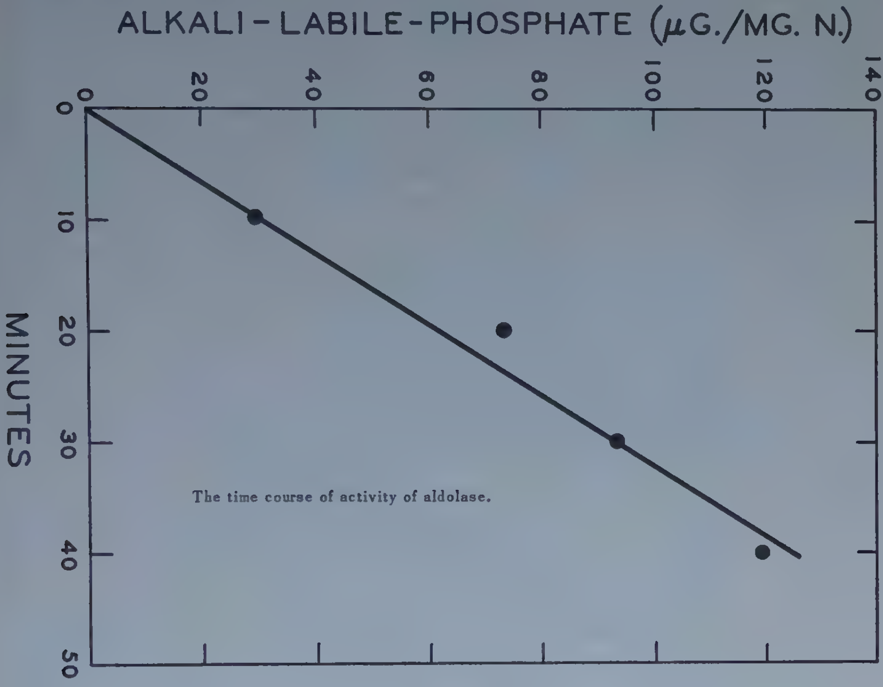
* Laboratory of Tropical Diseases.



The time course of activity of aldolase.

FIGURE 1.

TABLE 1

THE EFFECT OF HYDRAZINE ON THE AMOUNT OF COLOR PRODUCED (ISOMERASE TEST)

	Hydrazones D 540	*Aldehyde D 560*
T. cruzi homogenate series		
Incubated with hydrazine	0.077	0.091
Hydrazine added after TCA	0.281	0.507
Crystalline aldolase series		
Incubated with hydrazine	0.196	0.586
Hydrazine added after TCA	0.198	0.442

(2) *3-phosphoglyceraldehyde ⇄ dihydroxyacetone phosphate*

It is known that the ketose gives much more color per mole than the aldose, and therefore hydrazine must be present during incubation with homogenates to insure a 1:1 ratio of ketose to aldose. If hydrazine is omitted then isomerase

* We are indebted to Doctor B. L. Horecker for the crystalline aldolase used.

changes the ratio in favor of the ketose and more color will be obtained even with the same amount of splitting of hexose diphosphate. This effect can be seen from the data in the upper half of the table. The lower half shows that in the absence of isomerase, the omission of hydrazine has no effect on the amount of color produced in the hydrazone method. The data in the last column were obtained with the method of Dounce and Beyer (1948) and show essentially the same picture.

Complete characterization of triose phosphate dehydrogenase requires purified 3-phosphoglyceraldehyde as substrate, but this is difficult to obtain. Our studies have been carried out with hexose diphosphate as substrate, depending on aldolase and isomerase to supply the substrate for the dehydrogenase.

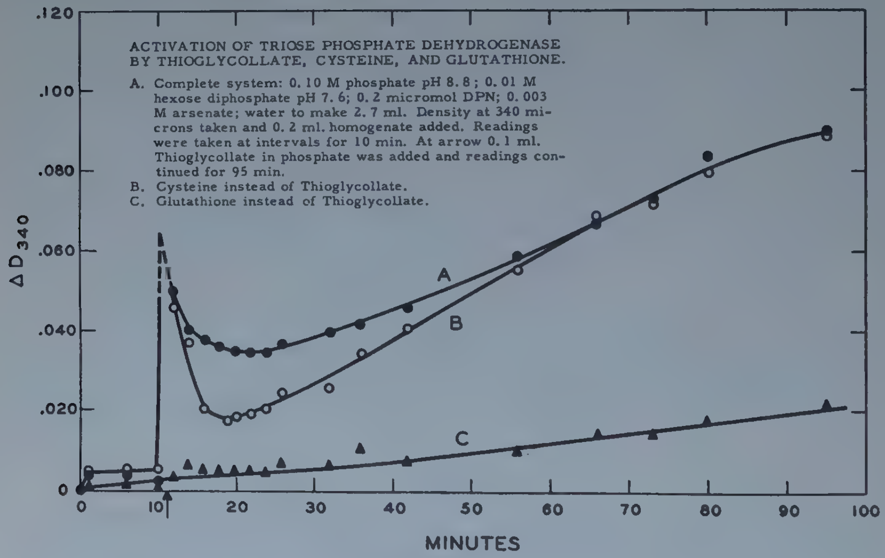


FIGURE 2.

During the preparation of the homogenate triose phosphate dehydrogenase is inactivated by oxidation of functional sulfhydryl groups and by dissociation of diphosphopyridine nucleotide which functions as a coenzyme.

(3) *3-phosphoglyceraldehyde + phosphate → 1,3-diphosphoglyceraldehyde*

(4) *1,3-diphosphoglyceraldehyde + DPN +* H_2O
→ *1,3-diphosphoglyceric acid +* $DPNH_2$

In order to study this reaction, the enzyme must be reactivated with cysteine, thioglycollate, or glutathione, and additional coenzyme added. The presence of arsenate prevents reversal by displacing one phosphate group in reaction (3) above. We have studied the reactivation process by following the increase in concentration of reduced DPN spectrophotometrically (FIGURE 2).

It is clear that triose phosphate dehydrogenase is present. The detailed characterization must await preparation of the purified substrate.

TABLE 2

RELATION OF KETO-ACID PRODUCED TO DIPHOSPHOPYRIDINE NUCLEOTIDE REDUCED

DPN ml.	$DPNH_2$ µmols	Oxalacetate µmols	$\frac{\text{Oxalacetate}}{DPNH_2}$
0.1	0.222	0.232	1.04
0.2	0.450	0.395	0.88
0.3	0.579	0.575	0.99
Average..			0.97

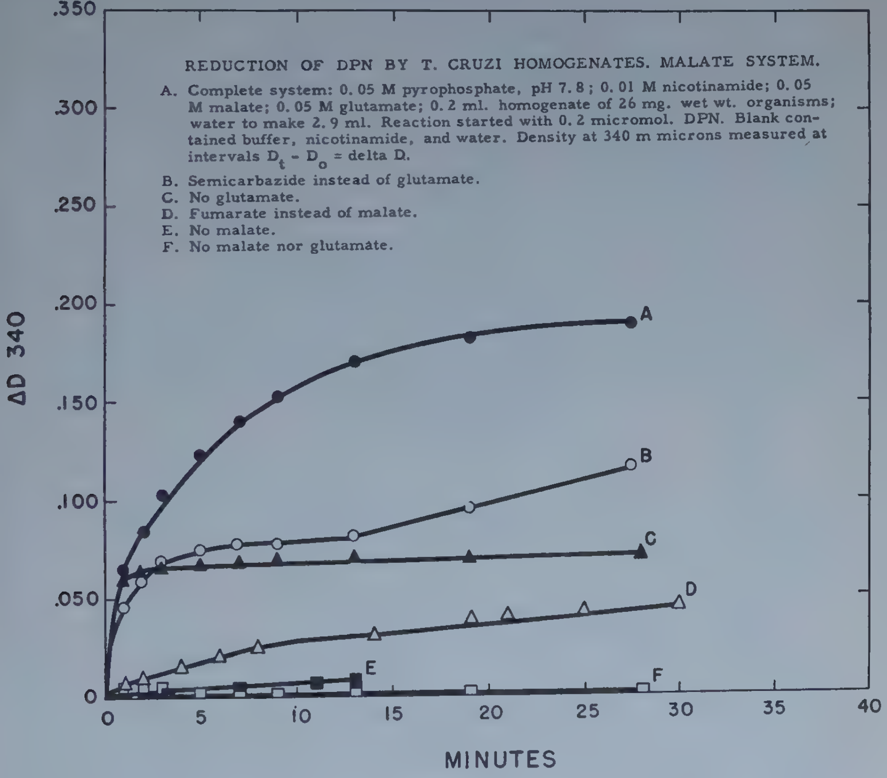


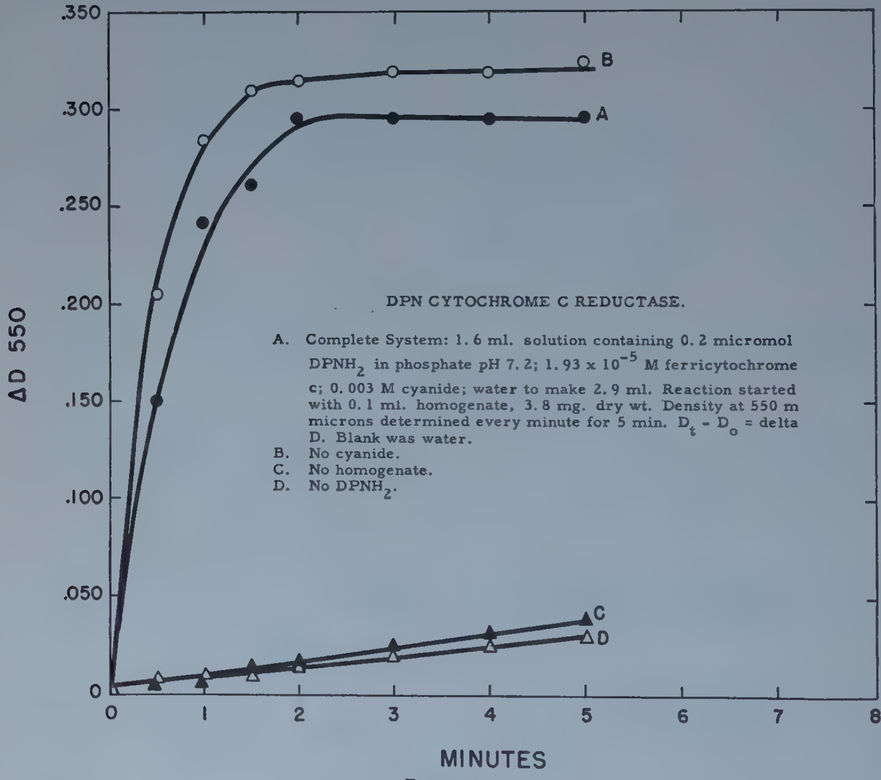
FIGURE 3.

One bit of evidence was obtained in addition, however, and that was the apparent absence of α glycerophosphate dehydrogenase. This enzyme has been found in most organisms studied, and it functions as a primer for the oxidation of triose phosphate by reoxidizing $DPNH_2$ using dihydroxyacetone phosphate for substrate.

TABLE 3
THE OXIDATION OF $DPNH_2$

Substrate	delta D_{340}
Oxalacetate	.202
Aspartate + keto glutarate	.204
Pyruvate	.010
Pyruvate + lactic dehydrogenase	.146

Mixtures contained 0.2 micromol $DPNH_2$ in phosphate pH 7.2; 0.01 M nicotinamide; 0.003 *M* substrate; water to make 2.9 ml. D_0 was determined and reaction started with 0.1 ml. homogenate. D_t was determined at 1 minute intervals for 9 minutes. Blank was water. D_0-D_t = delta D. Only final values are given.



DPN CYTOCHROME C REDUCTASE.

A. Complete System: 1.6 ml. solution containing 0.2 micromol $DPNH_2$ in phosphate pH 7.2; 1.93×10^{-5} M ferricytochrome c; 0.003 M cyanide; water to make 2.9 ml. Reaction started with 0.1 ml. homogenate, 3.8 mg. dry wt. Density at 550 m microns determined every minute for 5 min. $D_t - D_o$ = delta D. Blank was water.
B. No cyanide.
C. No homogenate.
D. No $DPNH_2$.

FIGURE 4.

(5) *Dihydroxyacetone phosphate* + $DPNH_2 \rightarrow \alpha$ *glycerophosphate* + *DPN*.

We found that when $DPNH_2$, prepared by chemical reduction, was added to the digest of hexose diphosphate and homogenate, no decrease in $DPNH_2$ occurred. This result indicates that α glycerophosphate dehydrogenase is inactive or absent. It would be interesting to test the ability of *T. cruzi* to metabolize glycerol, since this enzyme is required for oxidation after phosphorylation.

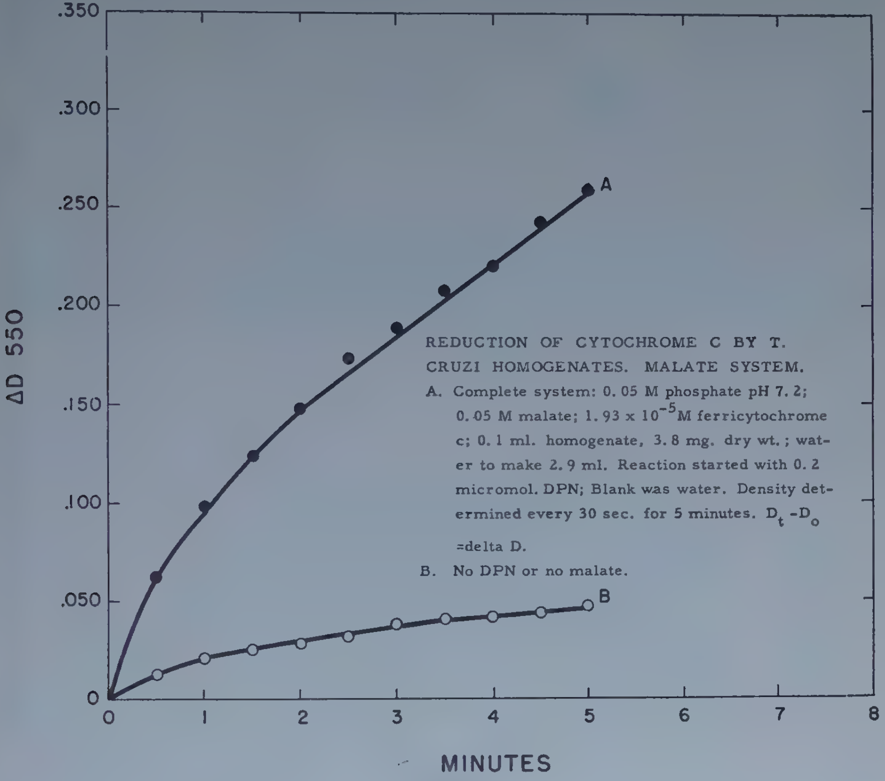


FIGURE 5.

TABLE 4

INDOPHENOL AND CYTOCHROME c REDUCTASE

pH	Indophenol Units/mg. N	pH	Cytochrome c Units/mg. N
7.4	43	6.7	11.3
7.8	64	7.0	12.5
8.2	56	7.3	13.8
9.1	27	8.1	10.0

Indophenol system contains: 1.0 ml. 0.15 *M* phosphate, 0.4 ml. 0.5 *M* malate, 0.1 ml. sodium 2,6, dichlorobenzenoneindophenol 3.5 mg./100 ml., 0.1 ml. undiluted homogenate containing 0.329 mg. N, water to make 2.6 ml. Reaction started with 0.4 ml. DPN 2.0 μmols/ml., pH determined at end of experiment. One unit indophenol reductase activity decreases log I_0/I 0.01 per minute at 600 mμ at 25°C. in a 1 cm. cell.

Cytochrome c reductase system contains: 1.0 ml. 0.15 *M* phosphate, 0.4 ml. 0.5 *M* malate, 0.4 ml. ferricytochrome c 0.791×10^{-4} *M*, 0.1 ml. undiluted homogenate containing 0.239 mg. N, water to make 2.9 ml. Reaction started with 0.1 ml. DPN 2 μmols/ml. pH determined at end of experiment. One unit cytochrome c reductase increases log I_0/I at 550 mμ 0.01 per minute at 25°C. in a 1 cm. cell.

No further reactions in glycolysis have as yet been studied save the attempt to demonstrate the reduction of pyruvate to lactate.

$$\text{Pyruvate} + DPNH_2 \rightarrow \text{lactate} + DPN. \qquad (6)$$

TABLE 5
FUMARIC HYDROGENASE

Complete system	Units
0.1 ml. homogenate	1.8
0.2 " "	3.7
0.3 " "	5.1
0.1 " " FAD 20 μg.	6.0
0.1 " " " 40 "	7.9
0.1 " " " 60 "	11.8
0.0 " " " 20 "	0.1
0.2 " " No fumarate	0.2

Homogenate was prepared from 0.4930 g. wet weight trypanosomes which had been frozen for 8 days. They were ground with 5.0 ml. water in a glass homogenizer, dialyzed over night at 4°C., and centrifuged 5 minutes at 4,300 rpm. The supernatant was separated and stored at 4°C. Aliquots were measured into the bulb of a Thunberg tube which contained 2.0 ml. Janus red 2 × 10^{-4} *M*, 0.1 ml. fumarate 0.5 *M*, and 0.8 ml. water. One drop of $Na_2S_2O_4$ solution was added to just decolorize the dye and the tube evacuated. The reaction was started by tipping in the enzyme and the rate of increase in optical density at 550 mμ determined in a spectrophotometer. One unit of activity changes the log I_0/I 0.01 per minute at 25°C. in a 1.0 cm. tube.

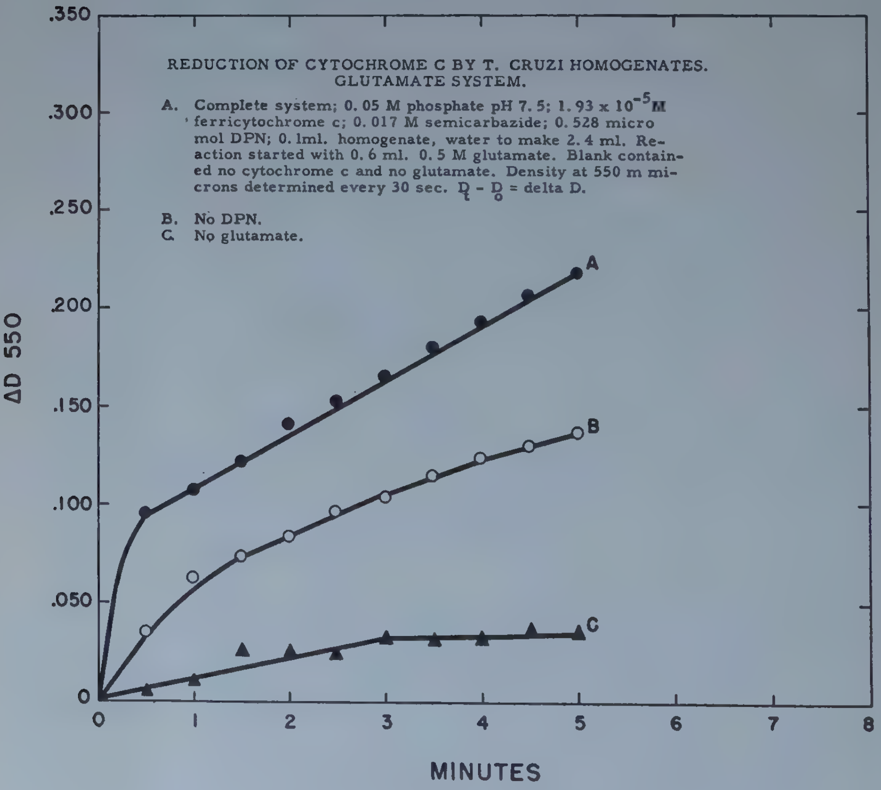


FIGURE 6.

Table 3 shows an insignificant oxidation of $DPNH_2$ when pyruvate was used as substrate. A control experiment with crystalline lactic dehydrogenase* instead of *T. cruzi* homogenate showed the expected reaction. Further experiments are planned to clarify the position of pyruvate in the metabolism of *T. cruzi*.

The oxidation of malate to oxalacetate is a prominent reaction of our homogenates. The reaction is reversible and the equilibrium favors formation of malate. This enzyme has been studied in considerable detail (Baernstein, 1952).

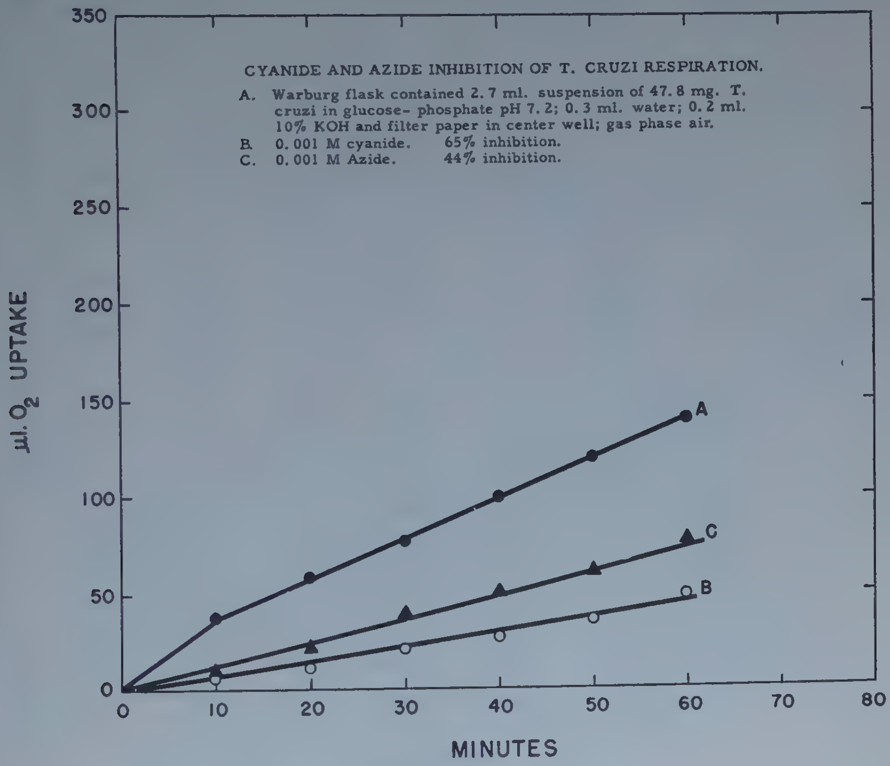


Figure 7.

$$(7) \qquad l\text{-}malate + DPN \rightleftarrows oxalacetate + DPNH_2$$

Table 2 shows that for each mole of DPN reduced one mole of oxalacetate is formed. Figure 3 shows that without semicarbazide or glutamate the oxidation of malate soon stops (curve C). This is usually attributed to inhibition by oxalacetate (Green, 1936). The relief of inhibition by glutamate has been attributed to transamination forming aspartate (curve A), (Cohen, 1951).

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* A gift from Doctor B. L. Horecker.

(8) *Glutamate + oxalacetate ⇄ ketoglutarate + aspartate*

This explanation is supported by the fact that aspartate and keto glutarate may be substituted for oxalacetate in the reverse reaction (TABLE 3).

When fumarate was substituted for malate, a slow reduction of DPN was observed. This shows the presence of fumarase, and this has been confirmed by the method of Racker (1950).

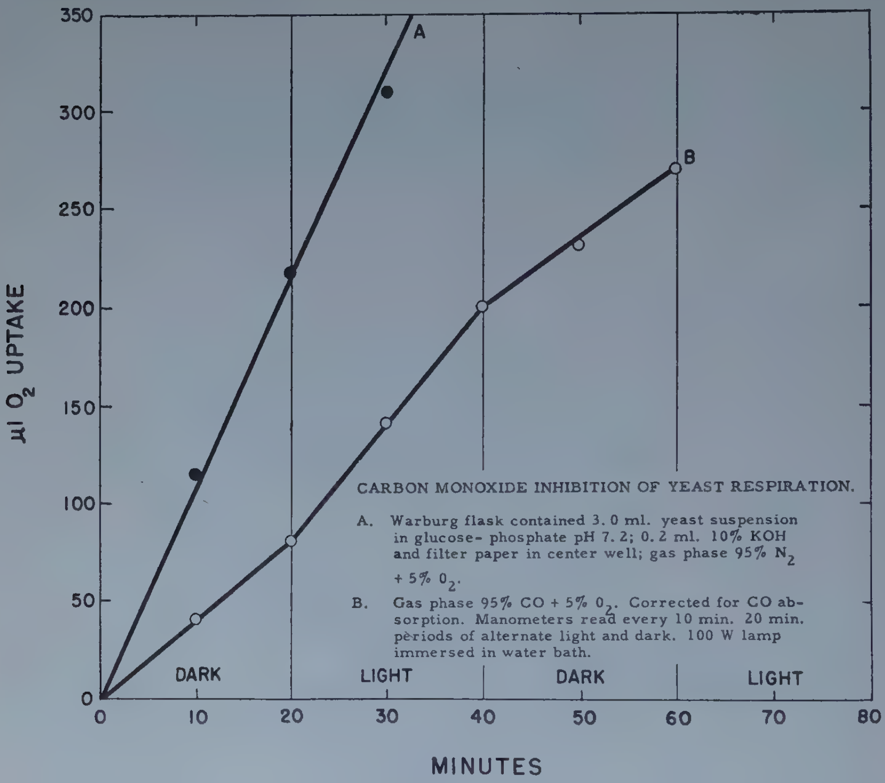


FIGURE 8.

(9) *Fumarate + water ⇄ malate*

Reduced DPN either by chemical (FIGURE 4) or enzymatic (FIGURE 5) means may be utilized to reduce cytochrome c or indophenol (TABLE 4). These reactions are probably mediated by flavoproteins.

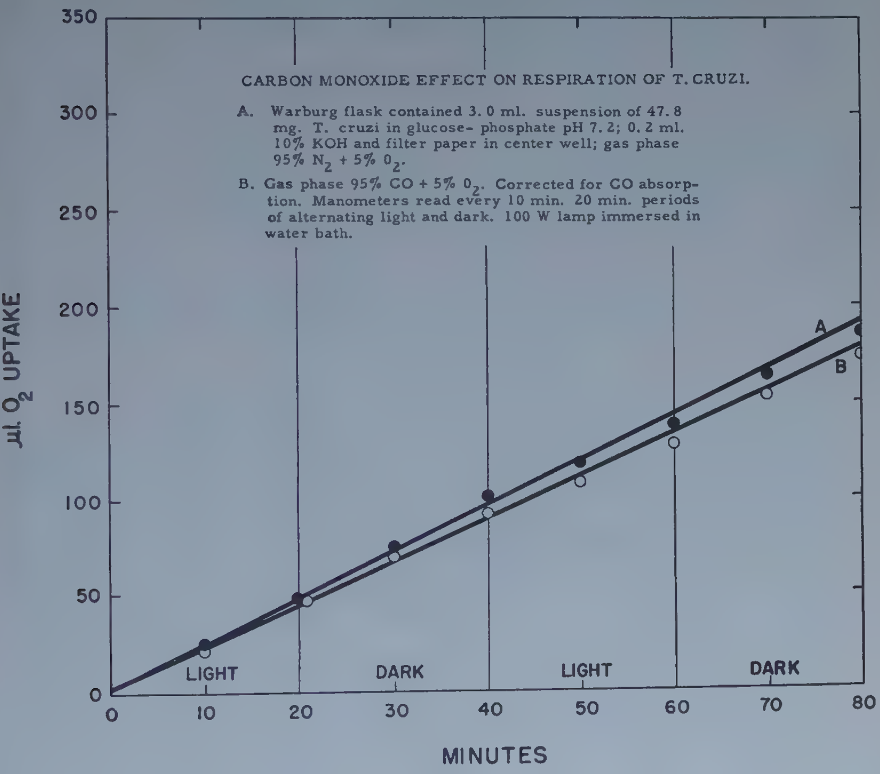
(10) $DPNH_2 + FAD \rightarrow DPN + FADH_2$

(11) $FADH_2 + indophenol \rightarrow leuco\ indophenol + FAD$ (*indophenol reductase*)

$$(12)\quad FADH_2 + \textit{ferricytochrome } c \rightarrow \textit{ferrocytochrome } c + FAD\ (\textit{cytochrome } c \textit{ reductase})$$

Still another type of flavoprotein activity found is the reduction of fumarate to succinate (Fischer and Eysenbach, 1937).

$$(13)\quad FADH_2 + \textit{fumarate} \rightarrow \textit{succinate} + FAD\ (\textit{fumaric hydrogenase})\ (\text{TABLE } 5)$$



CARBON MONOXIDE EFFECT ON RESPIRATION OF T. CRUZI.

A. Warburg flask contained 3.0 ml. suspension of 47.8 mg. T. cruzi in glucose- phosphate pH 7.2; 0.2 ml. 10% KOH and filter paper in center well; gas phase 95% N_2 + 5% O_2.

B. Gas phase 95% CO + 5% O_2. Corrected for CO absorption. Manometers read every 10 min. 20 min. periods of alternating light and dark. 100 W lamp immersed in water bath.

FIGURE 9.

Much more work is required before these enzymes can be discussed in detail. A potential metabolic pathway from aspartate to oxalacetate to malate to fumarate to succinate can be constructed with the enzymes just described. Whether this is the pathway utilized by *T. cruzi* must await further experiments.

A second link with protein metabolism besides transaminase is the DPN-linked glutamic dehydrogenase (FIGURE 6).

$$(14)\quad \textit{Glutamate} + DPN + H_2O \rightarrow \textit{ketoglutarate} + DPNH_2 + NH_3$$

In this case we measured the reduction of cytochrome c in the presence of glutamate and DPN. Further work on this system is indicated.

There is abundant evidence (Baernstein and Tobie, 1951) that *T. cruzi* contains a terminal oxidase which is cyanide and azide sensitive (FIGURE 7). Oxygen uptake was inhibited 65 per cent by 0.001 M cyanide and 44 per cent by 0.001 M azide. The terminal oxidase of yeast was shown by Warburg (1926) to be inhibited by carbon monoxide. The inhibition was reversed by light.

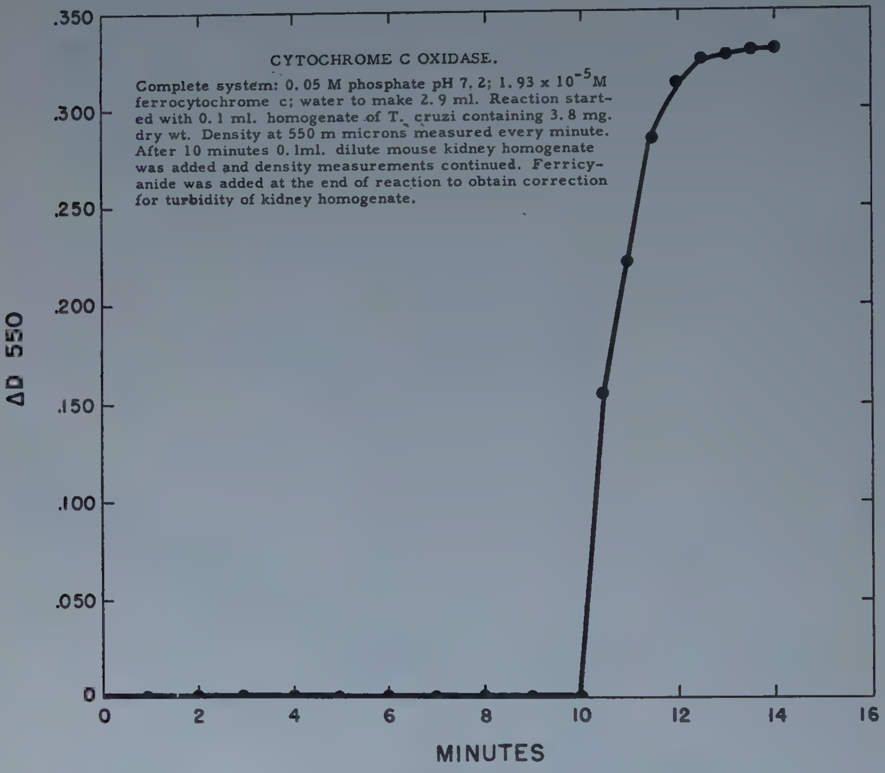


FIGURE 10.

TABLE 6

COMPARATIVE SPECTRA OF YEAST AND *T. cruzi*

	Millimicron values			
	a	b	c	d
Yeast	603	563	549	522
T. cruzi	604	556	—	532
	Pyridine Hemochromogens			
Protohemin	—	553	—	519
Yeast	—	556	—	530
T. cruzi	—	551	—	528

FIGURE 8 shows our confirmation of these results with yeast. FIGURE 9 shows the results of a similar experiment with *T. cruzi*. It can be seen that no inhibition of oxygen uptake occurred. This indicates that the terminal oxidase of *T. cruzi* differs from that of yeast in its sensitivity to carbon monoxide. These experiments should be extended to include higher ratios of carbon-monoxide to oxygen.

When ferrocytochrome c was mixed with our homogenate, no decrease in optical density at 550 mμ occurred indicating no cytochrome c oxidase activity (FIGURE 10). The introduction of mouse kidney homogenate led to an immediate and complete oxidation of cytochrome c. FIGURE 4 showed that in the reduction of cytochrome c by $DPNH_2$ the addition of cyanide was not necessary, showing again the absence of cytochrome c oxidase activity. TABLE 6 shows that when thick suspensions of *T. cruzi* are studied with a spectroscope no absorption band at 550 mμ is visible in reduced preparations. These observations indicate that the terminal oxidase of *T. cruzi* is linked to the cytochromes in a different manner than the oxidase of yeast. This subject deserves further study.

The absorption spectrum of the pyridine hemochromogen of *T. cruzi* does not differ significantly from that of protohemin.

Discussion and Summary

The presence of aldolase, isomerase, and triosephosphate dehydrogenase in homogenates of *T. cruzi* indicates a conventional glycolytic system. Two deficiencies may, however, exist since no evidence for α glycerophosphate nor lactic dehydrogenase could be found. The presence of pyruvate in the medium (Chang, 1948) supports the above hypothesis, but further work is necessary to establish the fate of this important metabolite.

Succinate is undoubtedly one of the chief end products of *T. cruzi* in culture. The enzymes malic dehydrogenase, fumarase, fumaric hydrogenase, and transaminase which we have found in homogenates may be organized to produce succinate anaerobically. This potential pathway requires that fumaric hydrogenase be reduced by $DPNH_2$, and this has not been demonstrated. An aerobic pathway for succinate production has not yet been found. The apparent absence of aconitase if confirmed would rule out the conventional Krebs cycle (Baernstein, 1952 in press).

The cytochrome system of *T. cruzi* needs considerable clarification. The apparent absence of cytochrome c as determined spectroscopically is supported by the finding that ferrocytochrome c was not oxidized by the cyanide-sensitive oxidase. Yet added ferricytochrome c can be reduced by $DPNH_2$. The failure of carbon monoxide to inhibit the oxidase in the dark should be confirmed. If true then the oxidase of *T. cruzi* differs significantly from the conventional cytochrome c oxidase.

It is obvious that a great deal more work is required before the metabolism of this organism can be properly understood, and still more is required before this information will be useful in establishing optimal conditions for growth in culture and for development of the vertebrate stages.

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AFRICAN SLEEPING SICKNESS TRYPANOSOMES: CULTIVATION AND PROPERTIES OF THE CULTURE FORMS*

By David Weinman
Department of Microbiology, Yale University, New Haven, Conn.

This paper deals with the African sleeping sickness trypanosomes, their cultivation and certain properties of the culture forms.

To those who are not working on this problem, the status of the cultivation of *Trypanosoma gambiense* and *Trypanosoma rhodesiense* may not be clear. Standard references state that cultivation can be achieved, but only irregularly —or that such a worker in such a year claimed to have grown one of these species. Any reference to current field work in Africa shows that the method is not used for diagnosis.[8] Thus, the net impression given is that successful cultivation is rather a rarity, perhaps achieved only with anomalous strains.

Actually, with the isolation medium which I will describe later, successful cultures of fresh strains can be made from very nearly 100 per cent of all infected laboratory animals and in the neighborhood of 70–80 per cent of all attempts at isolation; that is, repeated cultures may be necessary in perhaps 20–30 per cent of all infected animals. Furthermore, since these cultures can be maintained indefinitely by serial transfer, there is no difficulty in carrying these organisms in the laboratory for experimental purposes.

There are several media available, each with respective advantages and drawbacks. In general, isolation and maintenance media should be distinguished. Isolation from the animal will fail with certain media which are, however, perfectly satisfactory for subcultures, once growth has been established *in vitro*.

The medium with which I have the greatest experience is a blood nutrient agar slant with only three peculiarities: it contains human blood; the blood contains sodium citrate as an anticoagulant (ACD mixtures are unsatisfactory); the human blood is inactivated; and the red cells washed before incorporation in the medium.[7, 9] This medium has given me throughout four or five years of study a greater number of isolations than any other and, consequently, I shall refer to it as isolation medium.

Isolation medium may be indispensable for certain experimental work. For example, we are now working with a strain of *Trypanosoma gambiense* which could properly be called neurotropic. It is of low virulence and mice die about 12 months after inoculation, showing paralysis of the posterior train. Yet trypanosomes are extremely difficult to demonstrate by any of the usual methods. It may require 15, 20, or 25 blood examinations before the animal is proved to be infected. Inoculation of fresh animals is no help, for the same type of infection is reproduced. Yet the cultures readily detect infection and this is the only method which can be easily and successfully applied to the live animals.

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A second medium, devised by Tobie *et al.*, is made with rabbit blood and Locke's solution.[6] While not so satisfactory for isolation as the preceding, it is very suitable for work where maintenance is the principal requisite, since it gives high yields and organisms are easily harvested. Cultures which are obtained on isolation medium usually adapt themselves readily to growth on Tobie's medium, and the excellent service which the latter can render is clear to all who have read von Brand's many studies on these microorganisms.

One last remark on media concerns the very specialized procedure used by Yorke and his collaborators at the Liverpool School of Tropical Medicine for certain *in vitro* studies. Although sometimes called cultivation, this is entirely incorrect, and I am not aware that such a claim was ever made for it by Yorke or his coworkers. Yorke's method is essentially prolonged survival *in vitro* of the blood forms of the trypanosomes, an entirely different matter, as we shall see.

Thus far, I have dwelt upon the ease of cultivation. The question arises then—such being the case—why is the subject not as clear and simple as, for example, the cultivation of *Leishmania* or *Trypanosoma cruzi*?

One major reason is that many laboratories deal with strains which are non-cultivable with present techniques. Such strains have been reported in the past and, in my experience, there is no doubt whatever as to their reality. On at least two occasions, I have utilized strains which were maintained in laboratory animals by repeated passage. At the outset, these trypanosomes could be cultivated readily from the blood, but, as the number of passages increased, there came a time, somewhere after the 10th passage from *Glossina* (between 16 months and 3 years), when these previously cultivable strains would no longer grow upon the same isolation medium which previously had been highly successful.[9]

Both Roubaud and Duke have reported that old syringe-passaged laboratory strains also fail to develop in *Glossina*.[5, 2] Since the types which do develop and multiply in culture are also those which develop in *Glossina*, it seems quite possible that this failure to grow, either in the test tube or in the fly, are two manifestations of the same thing, a loss of ability to complete the normal cycle of development.

This is probably one more example of a protozoan life cycle which is fixed in nature, where the fixity is determined by the necessities of survival conditions, but is labile in the laboratory, where whole portions of the cycle drop out under the circumstances of artificial maintenance; in this case, repeated blood inoculations without fly passage.

This tendency for old laboratory strains to become uncultivable raises practical experimental difficulties. My own solution has been to maintain cultivable strains in dry ice storage at a temperature of -70°C., where they may be maintained for a period of years, and thus avoid the changes which come with animal passage.[10]

The question immediately arises as to whether there are non-cultivable strains in man. If, in the laboratory, strains become non-cultivable after a number of generations away from *Glossina*, then in a chronic infection in man, the same number of generations may easily occur, perhaps to be followed by the same loss of cultivability. This view assumes that it is the number of generations which is important rather than any method of selection by means of animal passage. I consider the view as unproved and the problem unsolved.

The second point I should like to discuss is the loss of infectivity of cultures. When blood from an infected animal is placed on isolation medium, growth becomes evident in 5 to 30 days, varying with the numbers of trypanosomes inoculated—5 days with inoculation of 10,000 organisms, up to 30 days with only 100.

These isolation cultures are non-infective. Likewise, all subcultures taken from them fail to infect. Infectivity *in vitro* is lost much more rapidly than we are accustomed to see with other Protozoa, and even with the majority of the bacteria.

This loss of infectivity is a problem which well merits investigation, since a solution would give us a clue to the nature of virulence, at least with this organism. Even more important, perhaps, it holds the promise of explaining a stage in evolution, of clarifying what the essential property of this insect flagellate was which made it a successful parasite of man and animals, in contrast to the other insect flagellates which never accomplished this feat.

Loss of infectivity of cultures has been remarked by other workers and one explanation has been widely and unjustifiably accepted. In cultures, it is said, those morphological types which are characteristic of the cycle in *Glossina* occur, and here I am in complete agreement. But, goes the argument, the fly cycle is never completed in culture. Specifically, metacyclic forms are not produced *in vitro* and, since these are the infectious forms, it is not surprising that cultures fail to infect.

This explanation is inadequate. Crithidia and metacyclic forms are produced in culture and loss of infectivity cannot be so explained.

Since this discussion rests in part on morphological grounds, FIGURES 1, 2, and 3 bear on the problem.

FIGURE 1, made from one of David Bruce's plates, shows the succession of forms of *T. rhodesiense* in *Glossina*. The elongate forms in the top row are characteristic of the proventricular types. In the next two rows are forms from the salivary glands, including some crithidia with kinetoplast anterior to the nucleus. These lead to the development of the stubby metacyclic forms seen in FIGURE 2, and it is these which are supposedly the final infectious forms for man and animals.

All of these can be duplicated from cultures. In FIGURE 3, row I, we have the most common forms observed in cultures, and you will notice the resemblance of certain of these to the proventricular forms in *Glossina*. These trypanosomes proceed to multiply actively and division takes place by duplication of the kinetoplast, then the nucleus, followed by division of the cytoplasm (FIGURE 3, rows II and IIIA). It is principally by means of division at these stages, that the culture population is maintained.

The significance of this next type requires analysis. Quite obviously, repeated divisions of the nucleus have taken place, there are numerous flagella present, but cytoplasmic cleavage is incomplete (FIGURE 3, II, the two last organisms). These forms have been seen before and have been interpreted by others as degenerative types. I show them here only to emphasize that identical multinucleate forms may be obtained from salivary glands of infected tsetse flies.

In FIGURE 3, IV A, B, and C, we come to the crux of the matter with culture trypanosomes which cannot be told apart from metacyclic forms obtained from a dissection of salivary glands of *Glossina*. Here, one group is from a salivary gland dissection; the other two from cultures of *T. rhodesiense* and *T. gambiense* respectively, and it is always an exercise of memory for me to remember which is which, since morphologically they are so similar. Actually, the last group with the μ scale is from *Glossina* (FIGURE 3, IV, C); the other two from cultures (FIGURE 3, IV, A and IV, B).

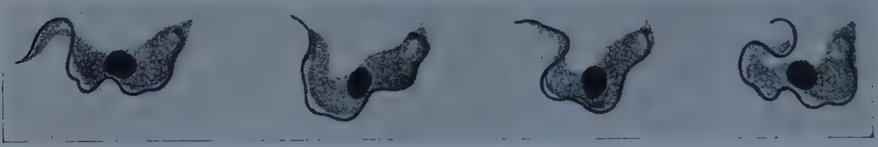

FIGURE 1.

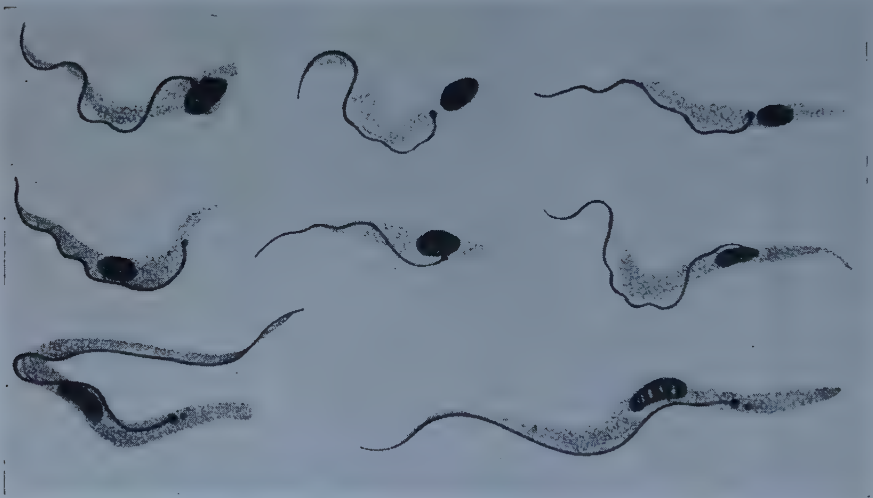

FIGURE 2.

We conclude from this that cultures do contain metacyclic trypanosomes or forms indistinguishable from them, and I may emphasize that it has never been doubted that the metacyclic form was a definite recognizable type. It follows, then, that loss of infectivity is not due to failure to complete the fly cycle *in vitro*; that is, it is not a qualitative failure in this morphological sense.

Is it a quantitative inadequacy, due to failure to inoculate a single infective dose of metacyclics? No final answer can ever be given to a question of this sort, since any dose can always be increased. But we can say that the number of metacyclics from *Glossina* known to be necessary for infection has been exceeded by massive inoculations of cultures without one single infection resulting.

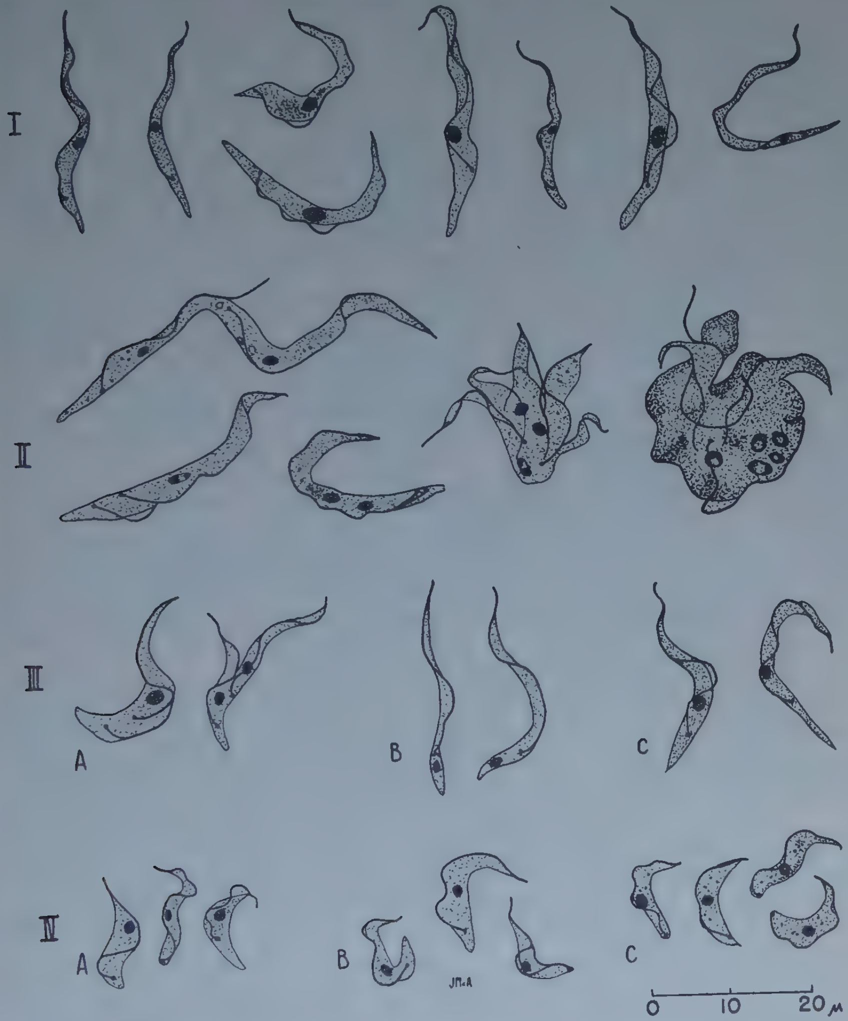

Figure 3. *Trypanosoma gambiense* and *T. rhodesiense*. Organisms from cultures, methyl alcohol — Giemsa, except as noted.

I. *T. gambiense*. Common cultural types.

II. *T. gambiense*. Single and multiple division forms in cultures.

III. *T. rhodesiense* from cultures. A) Dividing forms. B) Crithidia (osmium tetroxide vapor fixed.) C) Slender types with rod kinetoplast.

IV. Metacyclic type trypanosomes. A) *T. gambiense* culture. B) *T. rhodesiense* culture. C) From infected Glossina, dissection of salivary gland.

The conclusion seems very clear, that infectivity is a variable which fluctuates, and *in vitro* it can be independent of the completion of cyclical development and the production of forms which, in the fly, we associate with virulence or infectivity.

It follows, I think, from what has gone before, that a single strain of trypanosomes can continue in the laboratory in three different ways: First, the original strain obtained shortly after fly passage, which is inoculable and cultivable. This may be maintained as an animal passage substrain or as a culture substrain. The culture substrain is immediately non-infectious. The blood passage substrain sooner or later becomes non-cultivable. Thus, we end up with two derived strains which differ in main particulars.

This can be shown very clearly in the embryonated chick egg where the two strains are opposable in nearly every characteristic. For the blood passage strain, the embryo is an animal like any other. The important thing experimentally is to place the trypanosome in the blood stream or in the embryo. Then infection becomes established and the trypanosomes multiply.

On the contrary, with the culture trypanosomes, multiplication takes place only in the extra-embryonic fluids, and trypanosomes are not found in the embryo proper or in the heart blood. Different routes of inoculation are needed for the cultures, different temperatures of incubation, and younger, less differentiated eggs. After the culture trypanosomes have developed within the egg shell they still remain non-infectious both for the chick embryo and for other animals and, although they may be passed repeatedly in the allantoic fluid of fertile eggs or cultured on dead media, we have never had conversion to the blood type.

These data are summarized in TABLE 1.

A word is necessary, perhaps, as to why the cultures are termed non-infectious, although they do grow within the same egg shell as the growing chick. The important point, it seems to me, is whether invasion of the embryo, or its blood, or the tissues of the membranes takes place and, in this instance, it does not. That the allantoic and amniotic fluids are good culture media has no particular bearing on this point; as long as a living animal or tissue is not invaded, then true infectivity for an animal has not been achieved.

Several points which may be of interest have arisen in connection with our infectivity studies.

In the first place, cultures of *T. gambiense* grow very well under anaerobic conditions, at least on the isolation medium, any statements to the contrary notwithstanding. Not only is there luxuriant growth of the established cultures anaerobically, but it is possible to obtain the initial cultures, that is, directly from the blood of an animal, in an atmosphere of nitrogen. Since the culture trypanosomes grew abundantly in repeated anaerobic cultures, as did our control, the tetanus bacillus, it is probably a fair summary to state that culture trypanosomes are facultatively as anaerobic as the tetanus bacillus. This is probably another example of the difference between blood and culture trypanosomes which has already been shown, for example, by von Brand[1] in regard to the sensitivity to enzyme poisons.

The second point is that the trypanosomes usually do not grow at the body temperature of animals. This is not the factor which limits infectivity, however, for after many trials it was possible to grow the trypanosomes at 37° successfully, yet these organisms were no more infectious than any other.

Finally, Wigglesworth has shown that the midgut of *Glossina* contains the important digestive enzymes, that there is a strong trypsin-like substance and an active peptidase.[11, 12] We have exposed isolation culture medium to the action of pepsin, trypsin, and papain, but none of the degradation products provided by these enzymes produced infective trypanosomes, either on digested media or after transfer to fresh media.

The immunological study of cultures brought forth two points of interest. The first is that the cultures are antigenic and produce in mice agglutinins which readily reach titers of 1:1000 or more. Culture material which, you will recall, is not infectious, was then tried as an immunizing vaccine and tested against heterologous and homologous blood passage strains of high and low virulence respectively. The living cultures did not give rise to potent protective antibodies, for even when the experiment was organized to test resistance with very small infective doses of the low-virulent homologous strain, none of the "immunized" mice failed to become infected. Furthermore, there was not a significant delay in the incubation period as detected by cultures.

TABLE 1

Features of Egg Infection	Culture Trypanosomes	Mammalian Trypanosomes
Favorable age of embryo	Young (8 days)	Young or old
Optimal routes of inoculation	Intra-allantoic, Intra-amniotic	On chorioallantoic membrane, intravenous, intra-embryonic
Routes usually failing	On chorioallantoic membrane, intravenous	Intra-allantoic
Favorable temperatures of secondary incubation	32°C. or below	35°C. and above
Can be induced to multiply at	35°C. (after adaptation)	32°C. (adaptation may be necessary)
Loci of multiplication	Allantoic fluid Amniotic fluid	Embryo (blood, viscera)
Not found in	Embryo	Allantoic fluid
Repeated egg passage possible	Yes	Yes
Conversion to other type	No	No

The second point is the discovery of natural lysins in the blood of normal animals for the culture trypanosomes. If normal human or rabbit serum is placed in contact with culture trypanosomes, extremely marked lysis occurs. The lysin is active at 25° and 37°C. and the effect is still noticeable when the serum is diluted to 1:10.

Lytic activity can be greatly reduced by heating to 56° for 30 minutes or by the addition of heparin. Since complement is inactivated by these two procedures, it seems probable that the natural lytic substance requires complement or a complement-like substance for full activity.

What is the importance of these natural lysins? They may play some part in the failure of cultures to infect animals, but it has not yet been shown that they are decisive. Notably, the mouse appears deficient, partially at least, in this natural lytic substance; nevertheless, the mouse resists infection with culture trypanosomes quite as well as the rabbit. It may be pertinent to recall here that mouse serum is very nearly devoid of complementary activity when tested in the usual way.

The second point is the fact that the human, like the rabbit, possesses these natural lysins. In attempts to culture trypanosomes from human beings for diagnostic purposes, the results have thus far been disappointing. This may be readily explicable if lysins destroy the culture trypanosomes as they are formed.

This difficulty is a very real one and just recently we believe that we may have found a method for coping with it. There exist a number of well-defined chemical substances with anticomplementary activity. One of these is polyvinyl sulfuric acid, which has proved to have a number of virtues for our work. It has potent anticomplementary activity, for 0.5 mg. inactivates the complement in 1 cc. of guinea pig serum, and it is thus about 100 times as active as heparin.[3] It is an anticoagulant.

Most important of all, it appears to be non-injurious to trypanosomes in effective anticomplementary concentrations; so that the material can be added directly to the blood to be inoculated on medium. With this technique we have had better results than any obtained heretofore. For example, for the first time we have been able to use large blood inocula (1 and 2 cc. amounts) and have successful cultures, whereas control tubes inoculated with equal amounts of citrated blood were negative. Likewise, the minimal dose necessary for establishing cultures has been reduced through the use of PVSA. Plans are now made to check these laboratory results in man through field trials in Africa, where an attempt will be made to evaluate the whole cultivation procedure as a means of diagnosis and as a check on the effectiveness of chemotherapy.

Furthermore, it would seem that the use of PVSA may also be of value, not only in attacking certain of the problems of trypanosomiasis which I have mentioned, but may be of more widespread application in analyzing the role of complement in a great variety of infections with very different types of microorganisms.

I should like to terminate my paper by a brief reference to parallel studies in the bacterial field now being carried on in several laboratories, notably those of Ryan at Columbia, Szilard at Chicago, and Braun at Camp Detrick. These investigators have amply demonstrated the complexity of the microbial populations in growing cultures, and this with forms having a simpler life cycle than do the protozoa. We may well ask, for example, whether there are substances produced by our infective forms, or by the animal host, which influence the transition to non-cultivable or non-infective forms, with an effect similar to that of alanine on *Brucella* cultures.[4] These questions may be answered by a further analysis of the problem from the viewpoint of population dynamics and, if a chemical substance appears involved, by more extensive research directed toward the isolation and characterization of such factors.

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DISCUSSION OF PAPERS ON HAEMOFLAGELLATES

By Theodor von Brand
Federal Security Agency, Public Health Service, National Institutes of Health, National Microbiological Institute, Bethesda, Maryland*

The three papers on the growth of haemoflagellates showed how differently a great problem can be approached. This, unquestionably, is in part due to the predilection of a given investigator, but in part it is also due to the peculiarities of the experimental objects chosen. For example, it has been a general experience that certain Trypanosomidae, such as the strigomonads and the trypanosomes of the *lewisi* group, are quite easily cultivated, while the African trypanosomes are much more exacting in their requirements. It is, therefore, not surprising that the analysis of the former's growth requirements has progressed faster and farther than in the latter's case. The paper by Miss Cowperthwaite and her colleagues represents undoubtedly a milestone in our knowledge, inasmuch as her strigomonad is the first member of the Trypanosomidae cultivated in a completely synthetic medium.

It is probably not fortuitous that this significant advance was made with a strigomonad. Phylogenetically, it must be assumed that the Trypanosomidae were parasites of invertebrates before invading vertebrates, and, hence, it can be assumed that a pure insect parasite will represent a more simple case than that shown by a parasite alternating between various hosts. The invasion of vertebrates is correlated not only with the morphological changes with which we all are familiar, but also with physiological ones. As Doctor Weinman very justifiably has pointed out in his paper, much too little is known about the latter. But some data are known which make it quite clear that rather profound differences in metabolism exist between the culture forms (corresponding essentially to the insect forms) and the bloodstream form. This is true both for the *lewisi* group and the African trypanosomes. It is thus very easy to demonstrate a rather pronounced sugar consumption by the culture form of *Trypanosoma cruzi* while it has not been possible to do so in the case of its bloodstream form. Another example is *Trypanosoma gambiense.* The respiration of its bloodstream form is insensitive to cyanide, and its sugar degradation ceases at the pyruvate stage. The culture form of the same parasite, on the other hand, has a respiration that is at least partially cyanide sensitive, like that of all hitherto studied insect *Trypanosomidae*, and pyruvic acid is only a minor end product of its sugar metabolism.

It is quite possible, or even probable, that differences in metabolism between gut forms and salivary gland forms of the African trypanosomes could be found if it were possible to cultivate the latter. Doctor Weinman has found, in his cultures of *T. gambiense*, forms that appear morphologically indistinguishable from metacyclic trypanosomes. Their non-infectivity to vertebrates, nevertheless, shows that they are not true metacyclic trypanosomes physiologically, as Doctor Weinman himself has emphasized. Although the metabolism of the bloodstream form of the African trypanosomes appears, from a purely formalistic standpoint, in some ways simpler than that of the culture forms, there can hardly be a doubt that the absence of certain enzymatic reactions in the former represents not an original condition, but is due to the loss of certain functions. That is, we are dealing with a specialization rather than with an original simple condition. This specialization may well be the reason that it has, so far, proven impossible to cultivate any trypanosome in the form it has in the bloodstream of the definitive host.

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* Laboratory of Tropical Diseases.

Growth, obviously, is intimately tied up with a variety of metabolic processes. Nutrition, both in so far as macro- and micro-constituents of the diet are concerned, is one aspect. The intermediate metabolism of the ingested food is another. Both have an obvious direct bearing on the problem interesting us here; but I think it appropriate to point out that such studies have even wider implications, especially when applied to pathogenic organisms. I am thinking here of the implications such studies have on chemotherapeutic concepts. It has been shown repeatedly, for example, in malaria and in trypanosomiasis that the nutrition of the host has a rather profound influence on the course of infections. While it is true that the complicated host/parasite system rarely allows a definite answer to the question as to whether a certain nutritional deficiency or a certain supplement to the host's diet influences the growth of the parasites directly or indirectly *via* a subtle change in the physiological responses of the host, nevertheless it can be hoped that both *in vitro* and *in vivo* studies will lead to an elucidation of this elusive but intriguing problem. The preceding paper on the nutrition of *Strigomonas* may well serve as an example for future studies on pathogenic blood flagellates.

The studies on the intermediate metabolism of the ingested food, on the other hand, have a very direct bearing on the important question of how drugs affect the parasites. Modern chemotherapy shows more and more clearly that many chemotherapeutic agents act by interfering selectively with certain metabolic processes, often by inhibiting enzymatic activities of catabolic or anabolic nature. A chemotherapeutic agent will become of practical importance under one of the following conditions (aside, of course, from considerations of penetration into the parasite): (a) If the parasite relies for a vital function on an enzyme that the host does not possess; (b) if the parasite cannot bypass a blocked enzyme, but the host cell can; (c) if the parasites' enzyme, although performing the same catalytic activity as that of the host and being of the same importance, differs in its chemical reactivity from that of the host. It is clear that only a detailed study of the various enzymes of a parasite can decide which of the above, or perhaps other, possibilities are realized in a given case. Although the occurrence, in trypanosomes, of a number of enzymes was known for some time, a detailed study of their intimate relationships was so far lacking. Doctor Baernstein's presentation has shown convincingly that the material is amenable to such studies, and it can be expected confidently that this approach will yield information of both practical and theoretical importance.

STUDIES ON THE METABOLISM OF *TRICHOMONAS VAGINALIS*

By A. B. Kupferberg, H. O. Singher, G. Lampson, L. Levy, and A. H. Romano
Ortho Research Foundation, Raritan, New Jersey

Since the original isolation of a pure culture of *Trichomonas vaginalis* in 1939 by Trussell, studies on the metabolism of the organism have been very sparse, although some nutritional studies have been carried out.[1, 2, 3] In 1941, Trussell and Johnson[4] studied the fermentation activities of *T. vaginalis* on 32 carbohydrates and related compounds and demonstrated the ability of the protozoon to utilize glucose or a number of other polysaccharides composed of multiple glucose units. Fructose and galactose were also utilized but less effectively. Since that time, a requirement for inorganic phosphate and pantothenic acid has been demonstrated by Kupferberg *et al.*[3]

Johnson, in 1942,[5] demonstrated that increasing oxygen tension resulted in a decrease in population compared with those organisms maintained under strict anaerobiosis. Ascorbic acid was demonstrated by Johnson[6] to act as a stimulant for the growth of *T. vaginalis.* Since the advent of CPL medium in 1943,[7] however, and STS medium, in 1948,[3] which has the composition illustrated in FIGURE 1, no ascorbic acid has been employed, but the necessary redox potential in the medium has been maintained by the use of cysteine HCl. In the absence of added reducing agents, growth is extremely limited, thereby indicating the necessity for a low redox potential.

It has been shown by Suzuoki and Suzuoki[8] that *Trichomonas foetus* grown anaerobically produces lactic, pyruvic and succinic acid. *T. vaginalis* also produces acid in the course of its growth. In the routine STS medium, the initial pH is adjusted to 6 and sufficient acid is produced to shift the reaction of the buffered medium to approximately pH 5.2.

In order to investigate the nature of the acid produced, experiments were devised to measure lactic and pyruvic acid production. While some pyruvic acid was produced, it was in such minimal quantities that its effect on pH was negligible as compared to lactic acid production.

Lactic acid production. Lactic acid production was determined in a series of tubes, each of which contained 10 ml. of STS medium. The human serum normally employed was replaced by human serum that had been dialyzed 24 hours against water to reduce its lactic acid content. Each tube was then inoculated with 0.1 ml. of a 48 hour culture of *Trichomonas vaginalis*, strain #2, and incubated at 37°C. The pH and population counts were determined initially and at 4-hour intervals up to 48 hours on duplicate culture tubes. The pH was determined by glass electrode and population counts were made employing a haemocytometer. The residual portion of each individual culture was frozen immediately after removal of aliquots for pH and population determinations. Lactic acid was determined on these residual portions employing the method of Barker and Summerson.[9] The results obtained are shown in FIGURE 2.

It is apparent from this figure that in the initial 24 hours, when the population change was relatively small, there was only a small amount of lactic acid production and a concomitant small shift in pH. In the last 24 hour period, however, when the absolute change in population numbers was large, the change in lactic acid produced was also great with a proportionately large pH shift toward the acid side.

Composition of STS Medium

Trypticase (BBL)	20.0 gm.
Cysteine HCl	1.5 gm.
Maltose	1.0 gm.
Agar (Difco)	1.0 gm.
Distilled Water q.s.	95.0 ml.
Human Serum	5.0 ml.

Figure 1.

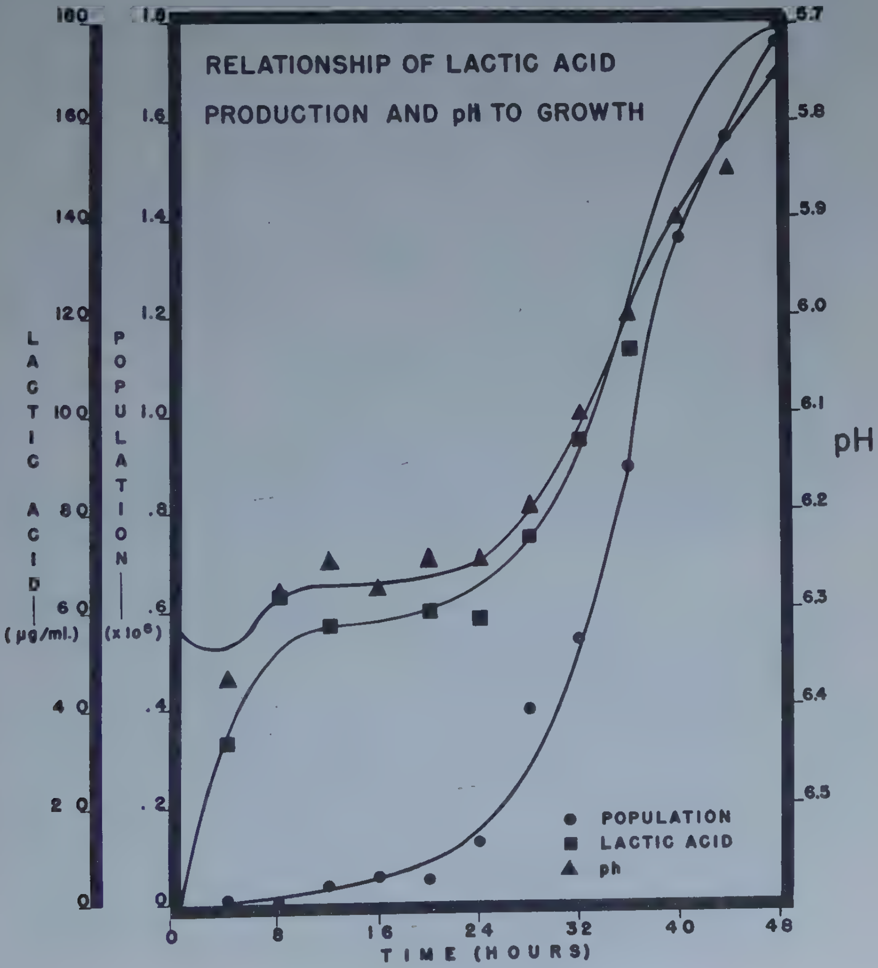
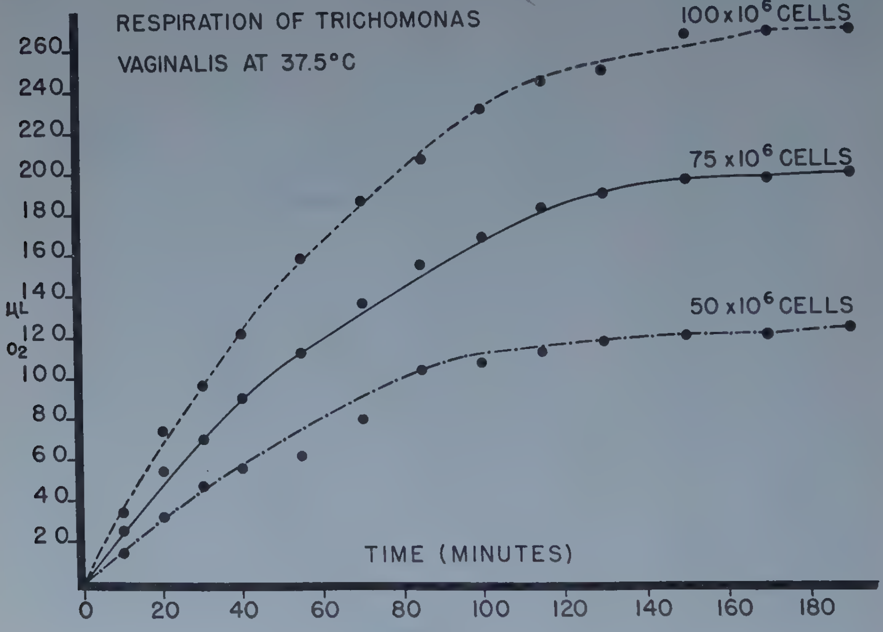


Figure 2.

The addition of lactic acid in amounts equivalent to that produced on incubation to uninoculated samples of STS medium produced pH shifts of the same magnitude. It is therefore apparent that the major acid produced by *T. vaginalis* in STS medium is lactic acid under the conditions necessary for its growth.

Respiratory metabolism. Despite the fact that *T. vaginalis* requires low redox-potential for growth and is capable of reproducing under anaerobic conditions, it has been possible to demonstrate that the organism can utilize oxygen in the presence of appropriate substrates.



FIGURE

Since Johnson reported, as mentioned previously, that increasing oxygen tension resulted in a decrease in population, it is likely that the respiratory activity is not essential for reproduction.

In order to facilitate the harvesting of the cells used in this study, it was necessary to omit agar from the medium and to grow the organisms anaerobically. In this manner, it was possible to obtain a concentration of organisms which was sufficient to exhibit measurable oxygen uptake.

FIGURE 3 represents the results of respiratory studies on *T. vaginalis* employing the usual Warburg technique, when oxygen uptake was measured in the presence of diluted STS medium, where glucose replaced maltose and the agar was omitted. The concentration of the glucose in the final solution was 1 per cent. It is clear from these curves that the magnitude of uptake was proportional to the number of cells.

Contents of Warburg Vessels:

0.2 cc. 20% KOH in center well; 0.3 cc. of 10% glucose in sidearms; 0.5 cc. of STS medium; cells and Ringer's carbonate to a total final volume of 3 cc.; gas phase: Air.

The oxygen uptake calculated from this and other experiments corresponds to an average uptake of 162×10^{-8} microliters of oxygen per cell/hr.

FIGURE 4 shows a comparative oxygen consumption of various parasitic protozoans. The data other than that of *T. vaginalis* was obtained from von Brand.[10]

As can be seen, the oxygen consumption of *T. vaginalis* is significantly lower than that of *T. foetus* and *T. hepatica*, but higher than that of the other parasitic protozoa listed.

FIGURE 5 represents the anaerobic glycolytic activity of *T. vaginalis* #2. In contrast to the experiments on production of lactic acid under the conditions of optimum reproductive activity, this study dealt with the essentially short term acid production of the organism. Since, under the conditions of this experiment, carbon dioxide evolution was representative of the formation of any acid stronger than carbonic, this curve represents total acid production rather than lactic acid alone. The average calculated production of carbon dioxide per 100 million organisms was 85 micro liters per hour. A survey of the field of parasitic protozoa did not reveal any data relative to carbon dioxide evolution under similar conditions.

OXYGEN CONSUMPTION OF SOME PARISITIC PROTOZOA

Organism	Oxygen Consumption mm³ O_2/100 million organisms/hr.
Trichomonas vaginalis #2	162
Trichomonas foetus	215
Trichomonas hepatica	600
Leishmania tropica	39
Trypanosoma cruzi	43
Plasmodium knowlesi	8–47

FIGURE 4.

Contents of vessels:

0.3 cc. 3*N* HCl and 10 per cent glucose in sidearms; 1 cc. STS medium without agar and without maltose; cells and Ringer's carbonate to a total value of 3.1 cc.; Gas phase: 95 per cent N_2, 5 per cent CO_2.

Similarly, a study was carried out on the respiratory quotient of the protozoon. FIGURE 6 depicts the oxygen uptake and carbon dioxide evolution that occurs under the usual experimental conditions. It will be noted that the ratio of carbon dioxide evolved to oxygen consumed decreased with time. Since the amount of substrate present was in a large excess, this change in the respiratory quotient with time must be explained in some other manner.

Oxygen Uptake and Carbon Dioxide Evolution.

Contents of vessels:

0.3 cc. 10 per cent glucose in sidearm; 1 cc. STS medium without agar and without maltose; For oxygen uptake: 0.2 cc. 20 per cent KOH in center well; cells and Ringer's carbonate to bring volume to 3.0 cc. Gas phase: Air.

FIGURE 7 is a representation of this change in respiratory quotient. Because of this change in R.Q., it is not possible to compare the respiratory quotient with that of other parasitic protozoa.

It seemed possible at this point that one explanation for the decreasing R.Q. might be the fixation of carbon dioxide. In order to investigate this possibility, studies were undertaken employing bicarbonate labeled with C^{14}. Preliminary experiments in the presence of radioactive bicarbonate indicated that fixation was occurring. It was then decided to attempt to determine the nature of the end product of carbon dioxide fixation.

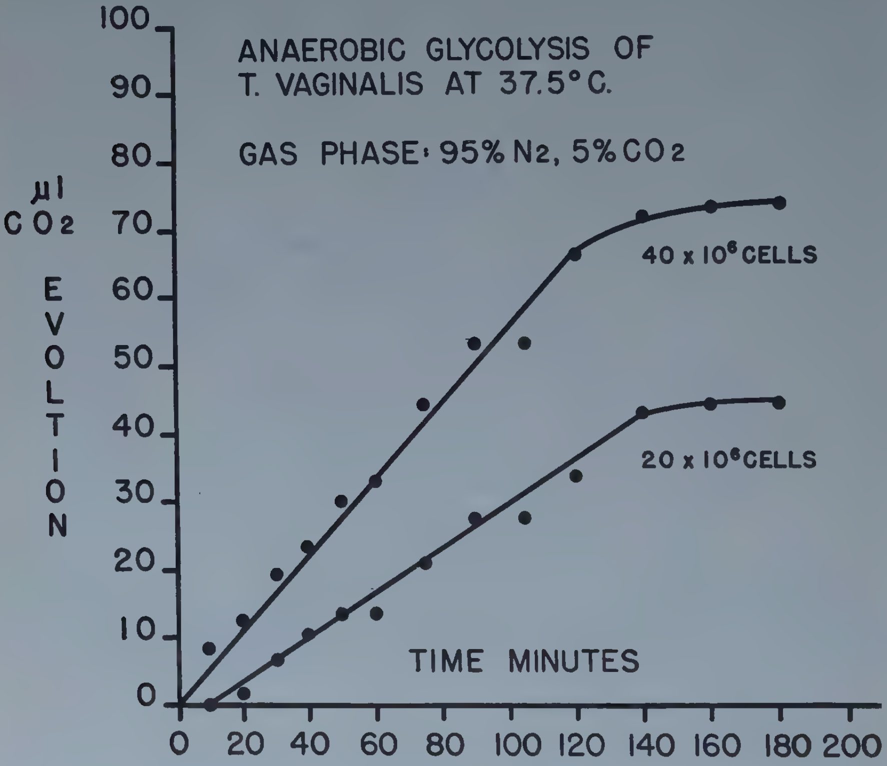


FIGURE 5.

Isolation and purification of carbon dioxide fixation product. For purposes of the radioactive study, all samples were oxidized by the Van Slyke wet combustion procedure.[11] Carbon dioxide was collected as barium carbonate, which was transferred in standard filtration assemblies to weighed aluminum discs. All samples were counted in regions equivalent to infinite thickness and corrected for background and scattering. Counts were made with a thin mica window-counter of less than 2 mgm./sq. cm.[12]

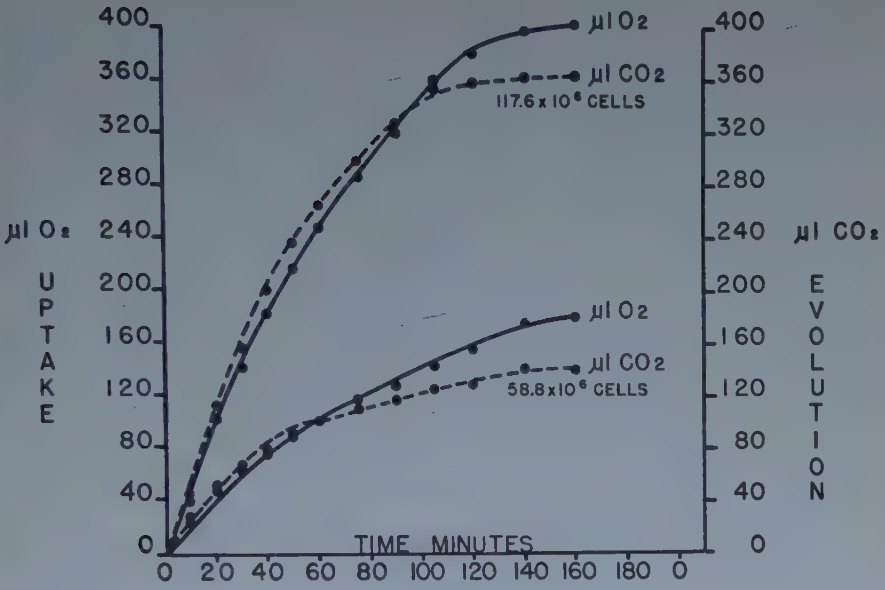


FIGURE 6.

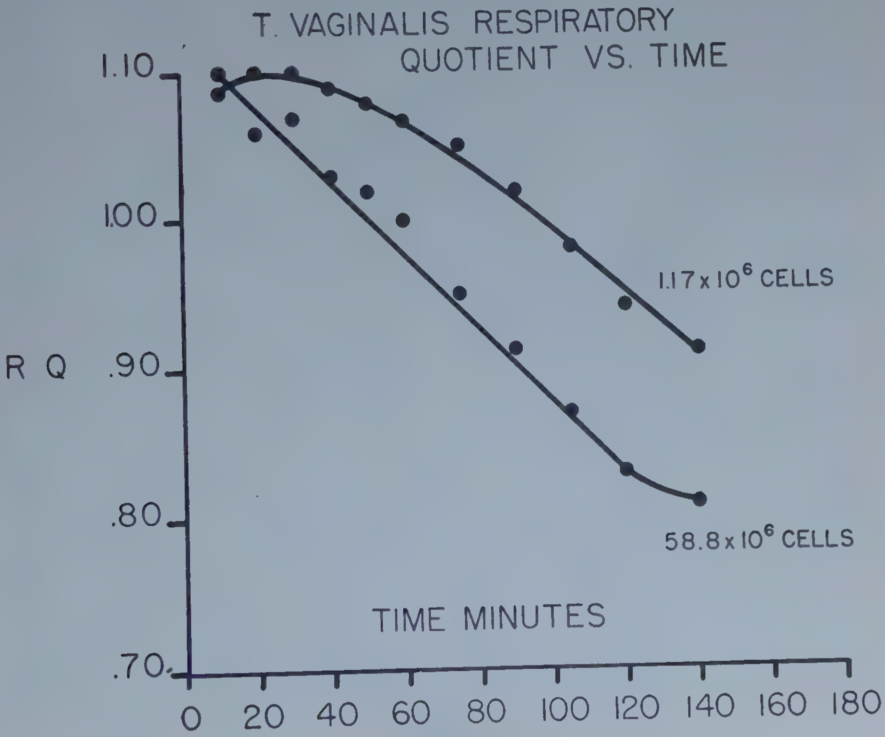


FIGURE 7.

Four liters of STS medium were prepared and adjusted to pH 6.8. The more alkaline pH was employed in order to maintain a greater concentration of bicarbonate in solution. 200 ml. of this medium was dispensed in 500 ml. bottles. The vessels were then plugged with cotton and autoclaved at 121° C. for 15 minutes. After cooling, the cotton plugs were removed and replaced with sterile rubber stoppers.

Adequate amounts of dialyzed sterile human serum adjusted to pH 6.8 were added to each of the above bottles to complete the medium. Finally, 25 ml. of sterile 0.005 M sodium bicarbonate containing enough radioactive bicarbonate to yield 1.4×10^4 counts per ml. per minute were added to each bottle. This level of radioactivity was chosen on the basis of preliminary experiments which proved that higher concentrations of C^{14} were deleterious to the growth of *T. vaginalis* #2. 1.25 ml. of a 48 hours culture of *T. vaginalis* #2 was then added to each bottle except the control. These were then incubated at 37°C. for 48 hours.

At the end of the period of incubation, microscopic examination revealed good growth. The filtrate and the protozoa were then separated by centrifugation and filtration (FIGURE 8).

After further centrifugation and filtration, microscopic examination of the filtrate failed to reveal the presence of any cells. Aliquots of both filtrate and precipitate of both experimental and controls were acidified and then treated with sodium tungstate. No radioactivity could be demonstrated in the controls or in the precipitates from the experimental. The supernatant resulting from the deproteinization of the filtrate contained all the residual measurable radioactivity.

Four volumes of cold absolute EtOH were then added to the radioactive supernatant and stored in the cold to allow precipitation to become complete. The precipitate and supernatant were separated. All the radioactivity remained in the supernatant. After removal of the EtOH by distillation *in vacuo*, the residue was concentrated by lyophilization. The dry powder was redissolved in 0.1 its original volume of distilled water and extracted with ether. The ether soluble material was found to be inactive and was discarded. The aqueous layer, which still contained the activity, was adjusted to pH 6.0 and barium was added in the presence of EtOH which resulted in the formation of a precipitate. The major portion of the radioactive material remained in the supernatant. The barium was removed by adding sulfuric acid. The supernatant had a high specific radioactivity. This material was examined for aldoses, ketoses,[13] and simple organic acids,[14] employing the technique of paper chromatography. Results were all negative. The material was found to contain nitrogen but no phosphorus. Ninhydrin reaction was positive but did not result in a loss of radioactivity, indicating that the C^{14} was not in the alpha carboxyl group of an alpha amino acid. Examination of this solution in the ultra-violet region revealed absorption maxima in the range of the aromatic amino acids, and the purine and pyrimidine bases. When the solution was chromatographed on paper, however, with a butanol-acetic acid system and examined with a mineral light lamp having a wavelength of 2537 angstrom units, which induces fluorescence in the purines and pyrimidines, the radioactivity occurred in the region that did not fluoresce. Similarly, when chromatograms were run either in a single dimension with S-collidine, or in two dimensions with S-collidine and isobutyric acids and developed with ninhydrin, the radioactivity could not be identified with any of the ninhydrin reactive areas.

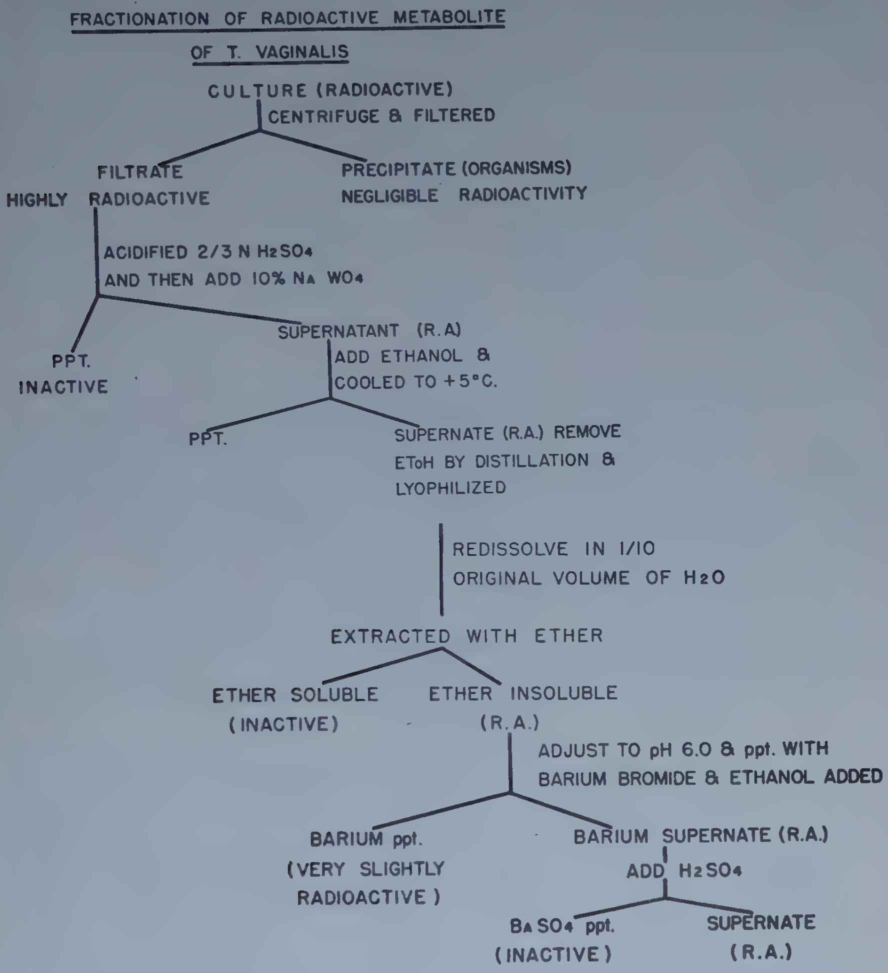

FRACTIONATION OF RADIOACTIVE METABOLITE OF T. VAGINALIS
CULTURE (RADIOACTIVE)
CENTRIFUGE & FILTERED
FILTRATE HIGHLY RADIOACTIVE
PRECIPITATE (ORGANISMS) NEGLIGIBLE RADIOACTIVITY
ACIDIFIED 2/3 N H2SO4 AND THEN ADD 10% NA WO4
PPT. INACTIVE
SUPERNATANT (R.A)
ADD ETHANOL & COOLED TO +5°C.
PPT.
SUPERNATE (R.A.) REMOVE EToH BY DISTILLATION & LYOPHILIZED
REDISSOLVE IN 1/10 ORIGINAL VOLUME OF H2O
EXTRACTED WITH ETHER
ETHER SOLUBLE (INACTIVE)
ETHER INSOLUBLE (R.A.)
ADJUST TO pH 6.0 & ppt. WITH BARIUM BROMIDE & ETHANOL ADDED
BARIUM ppt. (VERY SLIGHTLY RADIOACTIVE)
BARIUM SUPERNATE (R.A.)
ADD H2SO4
BA SO4 ppt. (INACTIVE)
SUPERNATE (R.A.)

FIGURE 8.

The radioactivity could then be eluted with absolute ethanol from the area in which radioactivity had been demonstrated. This procedure was carried out on multiple chromatograms.

The material obtained in this manner had no maxima in the ultra-violet range, and gave no reaction with ninhydrin. Its infra-red absorption spectrum presented maxima at points indicated in FIGURE 9.

This material gave no ninhydrin reaction or change in RF value after acid hydrolysis. It did not form a 2,4,dinitro phenyl hydrozone, nor a picrolonate.

Further characterization of the compound must be delayed until larger amounts of the radioactive material have been produced and isolated.

PROPERTIES OF RADIOACTIVE METABOLITE PRODUCED BY T. VAGINALIS

(A) No ninhydrin reaction
(B) No U.V. absorption
(C) No carbonyl or amine derivatives
(D) Infra-red absorption with maxima at
3.39 microns
3.45 microns
4.2 microns
4.3 microns
5.85 microns
6.0 microns
6.5 microns
6.8 microns

FIGURE 9.

Summary

(1) Lactic acid was found to be the major acid produced by *T. vaginalis.*

(2) The results of studies on the gaseous metabolism of *T. vaginalis* indicated that it had an average oxygen uptake of 162 microliters per 100 million cells per hour, an aerobic carbon dioxide evolution of 180 microliters per 100 million cells per hour, and an anaerobic carbon dioxide evolution of 85 microliters per 100 million cells per hour.

(3) Employing radioactive carbon, a CO_2 fixation product was isolated but not as yet identified.

In further studies, we have not been able to demonstrate the production of any gas other than CO_2 in any appreciable amounts.

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THE EFFECT OF RIBONUCLEIC ACID AND ITS NITROGENOUS CONSTITUENTS ON THE GROWTH OF *TRICHOMONAS VAGINALIS*

By Herbert Sprince, Raymond Goldberg, George Kucker, and Richard S. Lowy
General Microbiological Laboratories, Division of Elizabeth Bio-Chemical Laboratory, Elizabeth, N.J., and the Ortho Research Foundation, Raritan, N.J.

Very little is known about the nutritional requirements of parasitic protozoa. The isolation of pure cultures of *Trichomonas vaginalis* by Johnson and Trussell[1] prepared the way for investigation of the nutritional requirements of a parasitic protozoon highly fastidious in its growth requirements. As a starting point for such studies, a basal medium, containing, among other substances, three materials of natural origin (namely, BBL "trypticase," agar, and human serum) was devised by Sprince and Kupferberg.[2] Earlier studies by these investigators were concerned with serum fractionation[3, 4] and vitamin requirements.[5] Later studies were directed at the nature of the "trypticase" factor and its relationship to lipocaic (Lilly)[6] which was shown to be more effective than "trypticase." Since lipocaic is an extract from pancreas and as such is likely to be rich in nucleic acids or derivatives thereof,[7] it was felt that a detailed study of the growth stimulation or growth inhibition effects of ribonucleic acid (RNA), desoxyribonucleic acid (DNA) and the nitrogenous constituents thereof was now in order. It is the purpose of this paper to present the results of such an investigation.

Materials and Methods

The organism used. The organism used was a bacteria-free culture of *T. vaginalis* (strain no. 2) isolated by Johnson, Trussell, and Jahn in 1945.[8] It was carried in batteries of stock cultures at 37°C. in the basal medium described below (TABLE 1), to which 2 per cent "trypticase" was added.

Transfers of stock cultures were made on Mondays, Wednesdays, and Fridays, *i.e.*, during an interim of two 48-hour intervals and one 72-hour interval. For the 48-hour intervals, 0.07 ml. of inoculum (approximately 150,000 organisms) were used. For the 72-hour (week-end) interval, 0.05 ml. of inoculum (approximately 100,000 organisms) were used. Such a routine gave optimal results and prevented loss of cultures due to overgrowth in subsequent assay work.

The basal medium used. The original Sprince-Kupferberg medium[2] was simplified and, for purposes of this study, rendered basal with respect to purine and pyrimidine compounds. The composition of this medium is set forth in TABLE 1.

Procedure for assay. It should be noted that the following compounds were omitted in the preparation of the basal medium: "trypticase," adenine, guanine, uracil, and cytosine. In testing for the activity of various materials, the substrates under test were solubilized in the basal medium. The tubes were autoclaved, and the sterile serum was then added in the usual way. Two controls were always run concomitantly: one control contained 2 per cent BBL "trypticase;" the second control was the basal medium *per se.*

A routine was inaugurated wherein assays were begun on Fridays. The inoculum used for assay was prepared from a 48-hour stock culture (10 ml. tube) which was centrifuged aseptically, washed twice, and resuspended in sterile Ringer's solution (approximately 6–7 ml.). Inoculation was effected in the usual way by introducing 0.05 ml. (*ca.* 100,000 organisms) of the inoculum by pipette into the assay tube at the midlevel. Subsequent to this initial culture which was allowed to proceed for 72 hours, two consecutive 48 hour serial transfers, each with 0.07 ml. of inoculum, were made. At the end of the initial 72-hour period and again at the end of the second 48-hour serial transfer, a tenfold dilution of the tubes to be measured was made with Ringer's solution, and hemocytometer cell counts were then performed. Final data were expressed as number of cells per cubic millimeter. Duplicate determinations were made in each assay.

Table 1

Composition of Basal Medium

(*The amounts listed are the calculated values per 10 ml. of final volume*)

Sodium acetate·$3H_2O$	48 mg.	Pyridoxine·HCl	6.4 µg.
Cystine·HCl	15 mg.	Pyridoxamine·HCl	1.6 µg.
Maltose	10 mg.	Pyridoxal·HCl	1.6 µg.
Difco agar	10 mg.	Ca pantothenate	3.2 µg.
Asparagine	2 mg.	Nicotinic acid	3.2 µg.
KH_2PO_4	5 mg.	*p*-Aminobenzoic acid	1.6 µg.
K_2HPO_4	5 mg.	Biotin	0.8 µg.
Choline chloride	80 µg.	Pteroylglutamic acid	0.8 µg.
Inositol	80 µg.	Methylene blue (optional)	24.0 µg.
Ribose	40 µg.	Adjusted to pH 6.0 and Ringer's soln to make	9.5 ml.
Cryst. vitamin B_{12} (Merck)	0.1 µg.	Sterile human blood serum	0.5 ml.
Riboflavin	8.0 µg.	Final volume	10.0 ml.
Thiamin·HCl	1.6 µg.		

Precision of assay. It should be noted that in assays based on cell counts, an appreciable variation may be observed in duplicate determinations in a given assay and in repetitions of the same assay on different days. In view of this fact, all values reported herein are based on a minimum of two assays on different days; *i.e.*, quadruplicate determinations. Under these conditions, the normal variation which we encountered was between 20–30 per cent, a range well below that obtained (100 per cent or more) with materials of varying growth-promoting power.

Materials. The yeast ribonucleic acid and thymus desoxyribonucleic acid used in this work were obtained from the Schwarz Laboratories or the Nutritional Biochemicals Corporation. It was found that these preparations did not differ appreciably from highly purified preparations of yeast RNA (prepared by the procedure of Levene and Bass[9] and deproteinized by the method of Sevag, Lackman, and Smolens[10]) and DNA from thymus gland (prepared by the method of Mirsky and Pollister[11]). The ribonucleotides, and ribonucleosides, and the purine and pyrimidine bases were obtained from the following firms: Schwarz Laboratories; Nutritional Biochemicals Corporation; Ernst Bischoff Company; and the Eastman Kodak Company. It was found that materials obtained from any one of these dealers could be used interchangeably without any appreciable variation in results.

Hydrolytic procedures. To test the effect of mild acid and mild alkaline hydrolysis on the growth promoting activity of RNA and DNA, the following procedures were used:

(a) *Acid hydrolysis.* Five hundred milligrams of RNA or DNA were suspended in 25 ml. of $0.75N$ H_2SO_4 and refluxed in a boiling water bath for 2 hours. The hydrolysate was adjusted with $0.75N$ $Ba(OH)_2$ to pH 6.5 and all traces of Ba^{++} ion were removed. The $BaSO_4$ was filtered off and the filtrate then diluted to an appropriate volume for assay.

(b) *Alkaline hydrolysis.* Five hundred milligrams of RNA or DNA were suspended in 25 ml. of $0.75N$ $Ba(OH)_2$ and refluxed in a boiling water bath for 2 hours. The hydrolysate was adjusted with $5N$ H_2SO_4 to pH 6.5, and all traces of Ba^{++} ion were removed. The $BaSO_4$ was filtered off, and the filtrate then diluted to an appropriate volume for assay.

(c) *Enzymatic hydrolysis.* RNA was treated with ribonuclease according to the method of Volkin and Carter[12] and DNA with desoxyribonuclease according to the method of Volkin, Khym, and Cohn.[13] The DNA digest was carried up to the phosphatase stage only.

Results Obtained in Growth Stimulation Studies

In the data reported below, growth is expressed as the average number of cells/mm.^{3} of the number of determinations made. Measurement of growth at the end of the second 48-hour serial transfer is a more critical and reliable index of activity than readings obtained after the initial 72-hour period.

Effect of RNA, DNA, and hydrolysates thereof. The results obtained with ribonucleic acid (yeast) and desoxyribonucleic acid (thymus gland) are presented in TABLE 2.

It can readily be seen that in the absence of BBL "trypticase" no growth occurred. BBL "trypticase" (2 per cent) gave optimal growth in both the initial culture and at the end of the second 48-hour serial transfer. RNA could replace "trypticase" in supporting the growth of *T. vaginalis* up to a point, but as its optimal concentration of 0.1% RNA was not so effective as "trypticase" or lipocaic (Lilly). RNA became inhibitory with further increase in concentration. DNA would not support the growth of *T. vaginalis* at any of the concentrations tested. Addition of varying concentrations of DNA to the basal medium containing 0.01 per cent RNA resulted in mild growth inhibition during the initial 72-hour period, but growth eventually became normal as can be seen from counts at the end of the second 48-hour serial transfer. Acid hydrolysis of RNA definitely destroyed growth promoting activity, but alkaline and enzymatic hydrolysis (ribonuclease) had no effect. Neither acid, alkaline, or enzymatic hydrolysis (desoxyribonuclease) of DNA could render it capable of supporting growth.

Effect of monoribonucleotides. TABLE 3 presents data on the effect of monoribonucleotides on the sustained growth of *T. vaginalis.*

From Column II, it can be seen that a combination of the four acids, adenylic, guanylic, uridylic, and cytidylic at 0.01 per cent each was as effective as RNA at the same concentration. Of all the combinations of *three* nucleotides tested, only one, namely, adenylic, guanylic, and uridylic acids, was found to be as effective as the combination of all four compounds. Cytidylic acid could partially replace uridylic acid in the presence of adenylic and guanylic acids. Other combinations of *three* nucleotides could not maintain sustained growth. All possible combinations of *two* nucleotides were tried, but no growth could be obtained. Similarly, no single nucleotide was found which was capable *per se* of supporting growth.

TABLE 2

EFFECT OF RNA AND DNA AND HYDROLYSATES THEREOF ON THE SUSTAINED GROWTH OF *Trichomonas Vaginalis*

Material Assayed	Total No. of Detms.	Av. No. cells/ mm^3 at end of initial 72 hr. culture (I)	Av. No. cells/ mm^3 at end of second 48 hr. serial transfer (II)
Bsl *per se* (control)	20	0	0
Bsl + 2% BBL trypticase (control)	20	2200	2400
Bsl + 0.05% lipocaic (Lilly)	6	1830	1710
Bsl + 0.002% RNA	4	660	180
Bsl + 0.005% RNA	4	825	463
Bsl + 0.01% RNA	14	1450	660
Bsl + 0.05% RNA	8	1140	1039
Bsl + 0.1% RNA	4	2905	1315
Bsl + 0.5% RNA	4	1100	420
Bsl + 0.005% DNA	4	0	0
Bsl + 0.01% DNA	4	40	9
Bsl + 0.05% DNA	4	5	0
Bsl + 0.1% DNA	4	35	35
Bsl + 0.5% DNA	4	0	0
Bsl + 0.01% RNA + 0.01% DNA	4	780	560
Bsl + 0.01% RNA + 0.05% DNA	4	800	650
Bsl + 0.01% RNA + 0.10% DNA	4	770	610
Bsl + 0.01% RNA + 0.50% DNA	4	690	540
Bsl + 0.01% RNA (Acid Hy)	4	360	0
Bsl + 0.01% RNA (Alk. Hy)	4	1190	600
Bsl + 0.01% RNA (RNS Hy)	6	1330	560
Bsl + 0.01% DNA (Acid Hy)	4	80	0
Bsl + 0.01% DNA (Alk. Hy)	4	30	0
Bsl + 0.01% DNA (DNS Hy)	4	50	0

Legend:
Acid Hy = Acid hydrolysis
Alk. Hy = Alkaline hydrolysis
RNS Hy = Ribonuclease hydrolysis
DNS Hy = Desoxyribonuclease hydrolysis

Effect of ribonucleosides (TABLE 3). Curiously enough, sustained growth was markedly reduced when the nucleosides were used in place of those nucleotides found to support growth. The highest cell count obtainable was about one-tenth that obtained with the corresponding nucleotides. Even so, it is not unlikely that such a count might well have been the result of residual growth effects from a few hardy survivors of the original inoculum.

Effect of the purine and pyrimidine bases (TABLE 3). When the four purine and pyrimidine bases (adenine, guanine, uracil, and cytosine, 0.01 per cent each) were substituted in place of their corresponding monoribonucleotides or RNA, equivalent growth was obtained. The combination of *three* bases, adenine, guanine, and uracil, was almost equally as effective. However, other combinations of *three bases* resulted in markedly reduced growth or no growth at all after subculture. All possible combinations of two bases were found to be inactive. Likewise no single base was found capable of supporting growth.

TABLE 3

EFFECT OF RIBONUCLEOTIDES, RIBONUCLEOSIDES, AND THE PURINE AND PYRIMIDINE BASES ON THE SUSTAINED GROWTH OF *Trichomonas Vaginalis*

Material Assayed	Total No. of Detms.	Av. No. cells/ mm^3 at end of initial 72 hr. culture (I)	Av. No. cells/ mm^3 at end of second 48 hr. serial transfer (II)
Bsl *per se* (control)	8	0	0
Bsl + 2% BBL "trypticase" (control)	8	2010	2275
Bsl + 0.01% each (ADY 3, GNY, UDY, CDY)	10	1118	530
Bsl + 0.01% each (ADY 5, GNY, UDY, CDY)	12	1265	642
Bsl + 0.01% each (ADY 5, GNY, UDY)	4	1068	558
Bsl + 0.01% each (ADY 5, GNY, CDY)	4	1100	280
Bsl + 0.01% each (ADY 5, UDY, CDY)	4	465	0
Bsl + 0.01% each (GNY, UDY, CDY)	4	33	0
Bsl + 0.01% each (ADY 5, GNY)	4	15	5
Bsl + 0.01% each (ADS, GNS, UDN, CTD)	10	340	65
Bsl + 0.01% each (ADS, GNS, UDN)	4	310	50
Bsl + 0.01% each (ADS, GNS, CTD)	4	50	30
Bsl + 0.01% each (ADS, UDN, CTD)	4	80	0
Bsl + 0.01% each (GNS, UDN, CTD)	4	8	0
Bsl + 0.01% each (ADS, GNS)	4	5	0
Bsl + 0.01% each (A, G, U, C)	26	1378	639
Bsl + 0.01% each (A, G, U)	12	1029	410
Bsl + 0.01% each (A, G, C)	6	1288	90
Bsl + 0.01% each (A, U, C)	4	1620	90
Bsl + 0.01% each (G, U, C)	4	75	0
Bsl + 0.01% each (A, G)	4	15	10

Legend:
ADY = Adenylic acid
GNY = Guanylic acid
UDY = Uridylic acid
CDY = Cytidylic acid
ADS = Adenosine
GNS = Guanosine
UDN = Uridine
CTD = Cytidine
A = Adenine
G = Guanine
U = Uracil
C = Cytosine

Discussion. The results obtained in this study are of particular interest in view of the earlier findings of Skeggs, Spizizen, and Wright[14] that DNA was essential for the growth of *Lactobacillus bifidus* (ATCC 4963), and that DNA could be competitively inhibited by RNA. The inhibitory effect of RNA was later shown to be due chiefly to adenylic acid and to a lesser degree to guanylic, cytidylic, and uridylic acids, (Skeggs, Nepple, Spizizen, and Wright[15]). On the other hand, *T. vaginalis* responds solely to RNA; DNA has no effect; and adenylic acid or adenine must be present in any combination of nucleotides or bases essential for growth.

Since the medium contains human serum, the question may arise as to whether or not the nucleic acid content of serum is sufficient to interfere with the assay. Based on nucleic acid phosphorus determinations, Mandel and Metais,[16] have shown that human plasma normally contains between 35 and 52 mg. of nucleic acid per liter of which 90 per cent is ribonucleic acid and the remainder desoxyribonucleic acid. A simple calculation reveals that by adding 0.5 ml. of serum to a 10 ml. tube, the range of total nucleic acid contained in the medium is between 0.00018 per cent and 0.00026 per cent. From TABLE 2, it is evident that such concentrations would have negligible growth effects.

The data in TABLE 3 indicate that certain combinations of monoribonucleotides or purine and pyrimidine bases support the growth of *T. vaginalis*, an observation which was suggested by the hydrolytic findings on RNA (see TABLE 1). All four of the monoribonucleotides found in RNA or their corresponding purine and pyrimidine bases are necessary for *optimal* growth. At least two purines (adenine and guanine) and one pyrimidine (uracil) are necessary for consistently good growth. These results indicate that *T. vaginalis* is incapable of converting guanine to adenine for growth utilization as contrasted with *Tetrahymena geleii* (Flavin and Graff[17]) and *Lactobacillus casei* (Balis *et al.*[18]) where such a transformation has been shown to occur.

The inability of various combinations of nucleosides to support good growth in contrast to that ability manifest by the nucleotide combinations or their corresponding purine and pyrimidine bases (TABLE 2) has some interesting implications. Nygaard and Cheldelin[19] made a parallel observation with *Lactobacillus gayoni*. Contrary to the findings of these authors, increasing the phosphate concentration of our medium did not do away with the growth stimulation by nucleotides. Since the ribonucleosides seem to be almost inert in fostering growth of *T. vaginalis*, it is probable that they are incapable of being phosphorylated directly by the organism to the active monoribonucleotides. On the other hand, the free purines and pyrimidines are active. This may be by virtue of the attachment of phosphorylated pentoses to the free bases to give the active mononucleotides. Thus, it appears that monoribonucleotides essential for the growth of *Trichomonas vaginalis* can be formed from free purine and pyrimidine bases but not from their corresponding nucleosides. This is a point which has significent implications on the metabolism of purines and pyrimidines *in vivo* with respect to nucleic acid synthesis.

Results Obtained in Studies of Growth Inhibition

The inability of DNA or the monoribonucleosides to support the growth of *T. vaginalis* under conditions set forth in the preceding section led us to test these substances and other constituents of nucleic acids for inhibitory activity against growth promoting combinations of (a) the free purine and pyrimidine bases and (b) the corresponding monoribonucleotides. For these studies the basal medium presented in TABLE 1 supplemented with a combination of 0.01 per cent *each* of adenine, guanine, uracil and cytosine or a combination of 0.01 per cent *each* of the corresponding monoribonucleotides was used. Such concentrations were found to be optimal for growth. Substances or combinations of substances under assay for inhibitory activity were then added to the supplemented basal medium before autoclaving. Preliminary work soon indicated that DNA had very little, if any, effect against the free bases or the monoribonucleotides. The monoribonucleosides, however, were found to have considerable inhibitory activity. The results obtained with these substances are presented in detail below.

TABLE 4

INHIBITORY EFFECT OF NUCLEOSIDES ON THE GROWTH PROMOTING ACTIVITY OF THE PURINE AND PYRIMIDINE BASES ON *Trichomonas Vaginalis*

Material Assayed	Total No. of Detms	Av. No. cells/ mm^3 at end of initial 72 hr. culture (I)	Av. No. cells/ mm^3 at end of second 48 hr. serial transfer (II)
Bsl *per se* (control)	6	0	0
Bsl + 2% BBL "trypticase" (control)	6	2185	2040
Bsl + 0.01% each (A, G, U, C) (control)	6	1100	620
Bsl + 0.01% each (A, G, U, C) + 0.01% each (ADS, GNS, UDN, CTD)	6	1300	15
Bsl + 0.01% each (A, G, U, C) + 0.001% ADS	4	1020	120
Bsl + 0.01% each (A, G, U, C) + 0.005% ADS	4	1430	0
Bsl + 0.01% each (A, G U, C) + 0.01% ADS	6	1400	0
Bsl + 0.01% each (A, G, U, C) + 0.001% GNS	4	595	160
Bsl + 0.01% each (A G, U C) + 0.005% GNS	4	0	0
Bsl + 0.01% each (A, G, U C) + 0.01% GNS	6	0	0
Bsl + 0.01% each (A, G, U, C) + 0.001% UDN	4	890	460
Bsl + 0.01% each (A, G, U, C) + 0.005% UDN	4	1325	400
Bsl + 0.01% each (A, G, U, C) + 0.01% UDN	6	1010	240
Bsl + 0.01% each (A, G, U, C) + 0.001% CTD	4	1335	355
Bsl + 0.01% each (A, G, U, C) + 0.005% CTD	4	840	175
Bsl + 0.01% each (A, G, U, C) + 0.01% CTD	6	890	110

Legend:
A = Adenine
G = Guanine
U = Uracil
C = Cytosine
ADS = Adenosine
GNS = Guanosine
UDN = Uridine
CTD = Cytidine

Inhibitory effect of nucleosides on the optimal purine-pyrimidine combination. The inhibitory effect of the monoribonucleosides on the optimal combination of free bases is presented in detail in TABLE 4.

TABLE 4 demonstrates that a combination of adenosine, guanosine, uridine, and cytidine almost completely suppressed sustained growth (end of 2nd 48 hr. serial transfer). When tested singly, the order of inhibitory effectivity of the nucleosides was guanosine > adenosine > cytidine > uridine. Guanosine, alone, completely inhibited growth within 72 hours at a concentration of 0.005 per cent. All inhibitory effects could be shown to be non-competitive (irreversible) in nature.

Inhibitory effect of nucleosides on the optimal nucleotide combination. When the combination of free bases was replaced by the optimal combination of nucleotides, a similar inhibition occurred although the effect was not so great.

From TABLE 5, it is evident that the inhibitory effect of guanosine and adenosine is marked but not complete; whereas that of cytidine and uridine has all but disappeared. Inhibitory effects observed with guanosine and adenosine in this series were again found to be non-reversible.

TABLE 5

INHIBITORY EFFECT OF NUCLEOSIDES ON THE GROWTH PROMOTING ACTIVITY OF NUCLEOTIDES ON *Trichomonas Vaginalis*

Material Assayed	Total No. of Detms.	Av. No. cells/mm^3 at end of initial 72 hr. culture (I)	Av. No. cells/mm^3 at end of second 48 hr. serial transfer (II)
Bsl *per se* (control)	6	0	0
Bsl + 2% BBL "trypticase" (control)	6	2060	2010
Bsl + 0.01% each {ADY 5, GNY, UDY, CDY} (control)	6	1102	580
Bsl + 0.01% each {ADY 5, GNY, UDY, CDY} + 0.01% each {ADS, UDN, GNS, CTD}	6	510	110
Bsl + 0.01% each {ADY 5, GNY, UDY, CDY} + 0.01% ADS	6	1733	133
Bsl + 0.01% each {ADY 5, GNY, UDY, CDY} + 0.01% GNS	6	460	130
Bsl + 0.01% each {ADY 5, GNY, UDY, CDY} + 0.01% UDN	6	1235	440
Bsl + 0.01% each {ADY 5, GNY, UDY, CDY} + 0.01% CTD	6	1500	450

Legend:
ADY 5 = Adenylic (5) acid
GDY = Guanylic acid
UDY = Uridylic acid
CDY = Cytidylic acid
ADS = Adenosine
GNS = Guanosine
UDN = Uridine
CTD = Cytidine

Effect of replacing nucleosides by free bases or nucleotides. When, in TABLES 4 or 5, a given nucleoside at 0.01 per cent concentration was replaced by its corresponding free base or nucleotide at 0.01 per cent concentration, practically no inhibitory effect was noted with the exceptions of guanylic acid and adenylic acid derivatives. The replacement of guanosine by guanylic acid in 0.01 per cent concentration again resulted in almost complete growth inhibition within 72 hours and beyond. Since guanylic acid was added before autoclaving, however, it is possible that some guanosine may have been formed during the heating process. Oddly enough, however, replacement of adenosine by adenylic (3) acid, adenylic (5) acid, or adenosine triphosphate under the same conditions had only a very mild inhibitory effect.

Discussion. In the preceding section, it was suggested that growth of *T.*

vaginalis effected by free purine-pyrimidine bases may have been by virtue of the attachment to these compounds of previously formed phosphorylated pentoses to form the active nucleotides. This is in keeping with the recent findings of Kalckar,[20] Cohen and Scott,[21] and Horecker and Smyrniotis,[22] with regard to the enzymatic formation of pentose phosphate, and might account for the inactivity of the nucleosides to support growth.

On the other hand, the observation of the positive inhibitory effect on growth by nucleosides is a rather surprising one. Numerous reports have appeared in the literature to the effect that nucleosides may be stimulatory or relatively inert with respect to growth of various microorganisms; but this author knows of only one other instance where a nucleoside has been shown to have definite inhibitory effects on growth. Pulvertaft and Haynes,[23] in 1951, in connection with studies on spore germination showed that as little as 0.006 per cent of adenosine inhibited vegetative growth of *Bacillus cereus* and *Bacillus subtilis.* Interestingly enough, in our case as indicated above, 0.005 per cent of guanosine inhibited the growth of *T. vaginalis.*

Although it is premature to speculate at length concerning the above findings until they have been observed more generally, nevertheless, we would prefer to interpret our findings on *T. vaginalis* in the following light. The monoribonucleotides shown to be essential for growth can be formed by the living organism from their corresponding free nitrogenous bases but not from their nucleosides. In the former instance, nucleotide formation might occur by way of combination of free bases with enzymatically formed pentose phosphate. The inactivity of the nucleosides might be due to the inability of the organism to phosphorylate them to the active nucleotides.

The inhibitory effect of the nucleosides, on the other hand, leads us to wonder whether these compounds may not be regulatory in nature serving as natural inhibitors of the growth stimulating nucleotides or free bases. It is not unlikely that, in the initial stages of cell division, nucleosides may arise *in vivo* from nucleotides as a result of transphosphorylation reactions believed to occur in connection with nucleic acid metabolism and protein synthesis.[24, 25] Under conditions where the nucleotides or free bases are the limiting factors for growth, the nucleosides thus formed might then assume regulatory functions by virtue of inhibitory activity. Should such an antagonism, between nucleosides on the one hand and free bases and nucleotides on the other, prove to be prevalent in living organisms generally, far-reaching repercussions in the biochemistry of cell division would soon be forthcoming.*

Summary

Yeast ribonucleic acid (RNA), but not thymus desoxyribonucleic acid (DNA), could partially replace BBL "trypticase" or lipocaic (Lilly) in a modification of the Sprince-Kupferberg medium for the sustained growth of *Trichomonas vaginalis.* The activity of RNA was retained by alkaline or enzymatic (ribonuclease) hydrolysis, but was destroyed by acid hydrolysis. Neither

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* With reference to the above, it is of interest to cite the experiments of Parsons, Gulland, and Barker[26] who found that, in mouse tumors, the nucleotides, adenylic and guanylic acids inhibited growth, whereas cytidylic acid had little effect and uridylic acid even promoted growth.

acid, alkaline, nor enzymatic (desoxyribonuclease) hydrolysates of DNA could stimulate growth.

Sustained growth, equivalent to that with RNA could be obtained by a combination of the monoribonucleotides (adenylic, guanylic, uridylic, and cytidylic acids) or their respective nitrogenous bases (adenine, guanine, uracil, and cytosine), but not by their corresponding ribonucleosides (adenosine, guanosine, uridine, and cytidine). This was taken to indicate that *T. vaginalis* can convert the free purine and pyrimidine bases, but not their corresponding nucleosides, to the monoribonucleotides essential for growth.

At least three monoribonucleotides, adenylic, guanylic, and uridylic acids or their corresponding free bases are essential for consistently good growth of *T. vaginalis*. In contrast to *Tetrahymena geleii* and *Lactobacillus casei*, *T. vaginalis* is incapable of converting guanine to adenine for growth utilization.

The ribonucleosides could be shown to have a positive inhibitory effect against growth promoting combinations of (a) the free purine and pyrimidine bases and (b) the corresponding monoribonucleotides. When tested singly, the order of inhibitory effectivity of the nucleosides *per se* was guanosine > adenosine > cytidine > uridine. Guanosine alone completely inhibited growth within 72 hours at a concentration of 0.005 per cent. All inhibitory effects could be shown to be non-competitive (irreversible) with respect to the bases or nucleotides.

It was pointed out that, under certain conditions, a natural antagonism may exist between ribonucleosides on the one hand and free bases and nucleotides on the other. Under such conditions, the nucleosides would act as natural inhibitors of the growth stimulating nucleotides or free bases and thus serve as metabolic regulators of cell division.

Addendum

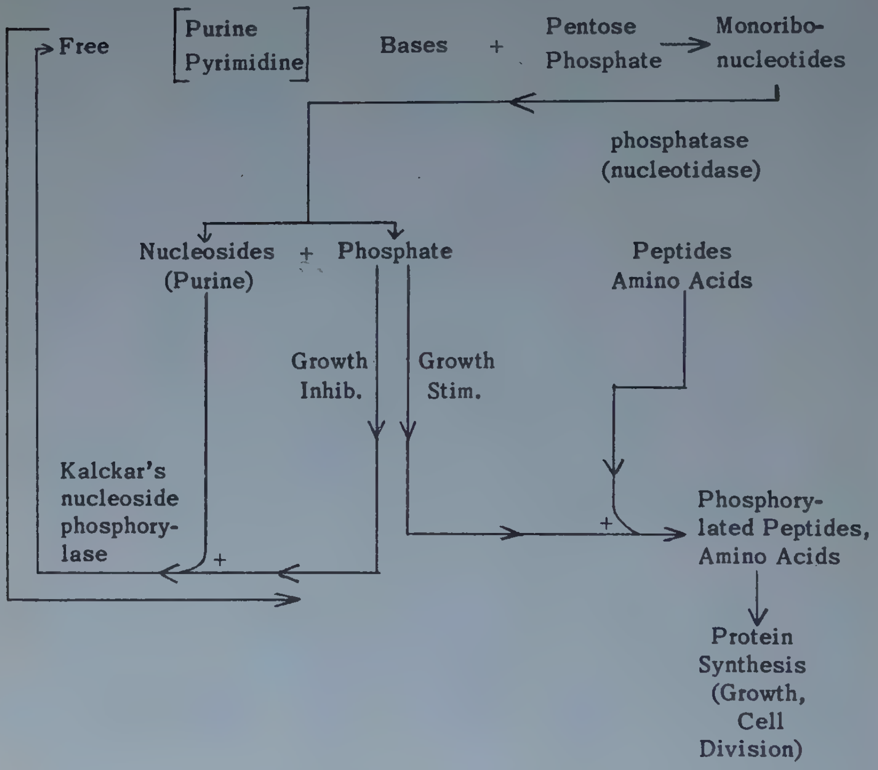
It is important to note that the inhibitory effect of certain nucleosides on the growth of *T. vaginalis* as presented in the foregoing paper was demonstrated in a medium *markedly low* in peptide and/or amino acid content (0.5 ml. of serum per 10 ml. final medium, See TABLE 1, above). The question now arises can such an inhibitory effect be shown to occur in a medium *rich* in peptides and/or amino acids?.

To answer this point, the effect of nucleosides on the growth of *T. vaginalis* was tested in the basal medium enriched with 2 per cent BBL "trypticase." With such a medium, a combination of 0.01 per cent each of adenosine, guanosine, uridine, and cytidine showed no growth inhibition whatsoever. A combination of 0.05 per cent each of the same nucleosides gave evidence of only partial inhibition. Concentrations of the individual nucleosides ranging from 0.01–0.05 per cent showed signs of partial inhibitory activity only at the higher concentrations as was to be expected.

Although the quantitative aspects of the reversal of nucleoside inhibition by "trypticase" in the presence or absence of combinations of nucleotides or free bases have yet to be worked out in detail, it is probably safe to say that growth inhibition by nucleosides does *not* take place in a medium *rich* in peptides and/or amino acids (Assuming, of course, the "trypticase" effect to be due to its high peptide and amino acid content).

FIGURE 1 summarizes possible relationships growing out of these findings.

A WORKING GUIDE FOR RELATIONSHIPS BETWEEN GROWTH, NUCLEIC ACID METABOLISM, AND PROTEIN SYNTHESIS IN *TRICHOMONAS VAGINALIS*



Nucleosides inhibit growth by competing with peptides and amino acids for phosphate.

FIGURE 1.

The above diagram would explain:

(1) why in a medium *low* in peptide and/or amino acids, addition of nucleosides *apparently* antagonizes growth stimulation by nucleotides and free bases.

(2) why a medium *rich* in peptides and/or amino acids overcomes the inhibitory effect on growth of added nucleosides.

In conclusion, perhaps, it should be indicated, that the one significant point which emerges from these findings is the possible rôle of (purine) nucleosides (and concomitantly of nucleoside phosphorylase) as metabolic regulators of cell division by virtue of limiting the amount of phosphorus made available for protein synthesis.

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STUDIES ON THE CULTIVATION OF *ENDAMOEBA HISTOLYTICA* WITH *TRYPANOSOMA CRUZI*

By Bruce P. Phillips

Federal Security Agency, Public Health Service, National Institutes of Health, National Microbiological Institute, Bethesda, Maryland*

Although amoebic infection as a distinct disease has been recognized since 1875, the effective study of the disease and of the organism which produces it has been made difficult by an inability to cultivate the causative organism, *Endamoeba histolytica*, in pure culture. Since the first really successful cultivation of this amoeba by Boeck and Drbohlav in 1925,[1] there have been numerous contributions to our knowledge of cultural procedures. Dobell and Laidlaw[2] observed that the addition of rice starch to cultures greatly enhanced the growth rate of the amoebae. These, and other results improved the cultural procedures and thus provided for greater production of amoebae *in vitro.* The general realization, however, that such cultures, which were predominantly bacterial cultures, could not be employed in studies on the physiology of the amoebae, on serodiagnosis, chemotherapy, and pathogenesis of the disease, stimulated a change in emphasis of cultural investigations. There was increased interest in the bacterial associates, or specifically, in the elimination of bacteria from such cultures.

Cleveland and Sanders[3] successfully transferred sterile amoebae from liver abscesses in cats to sterile culture medium, but the amoebae failed to grow. Meleney *et al.*,[4] and Snyder and Meleney[5] employed chemical treatment as a means of sterilizing cysts for use in cultural investigations. Rees and associates[6, 7] made effective use of microisolation to obtain sterile cysts for such studies. Thus, methods were devised whereby sterile amoebae could be obtained for studies on their requisite growth factors. Although excystation, however, occurred in various sterile environments[4, 5] the amoebae failed to grow.

Resulting, however, from a continuation of these studies, there began a series of accomplishments, all closely related, which effected considerable improvement in methods of cultivating the amoeba. In 1939 and later, Rees *et al.*[6, 8, 15] with the aid of a micromanipulator, established cultures of the amoeba with various single species of bacteria and published a list of these species and of various species with which the amoeba failed to excyst and grow. The abundant growth of amoebae with a bacterium, designated as organism *t*, and with *Clostridium perfringens* suggested that the amoebae did not require the vigorous activities of a rapidly-growing mixed bacterial flora such as had characterized most previous cultures of amoebae. Coincidently, the development of antibiotics, along with these observations led to an effort to cultivate the amoeba with inhibited bacterial associates. Jacobs, in 1947,[9, 10] demonstrated the usefulness of antibiotics in eliminating bacteria from amoeba cultures and provided thereby a basis for studies on growth requirements. Shortly thereafter, in 1948, Shaffer and Frye[11] and Shaffer *et al.*[12] utilized antibiotics for the elimination of bacteria from cultures of the amoeba and investigated the ability of antibiotic-inhibited bacteria to support growth of amoebae. These investigations led to the development of a cultural procedure whereby abundant growth of amoebae was produced in liquid medium with a non-multiplying, penicillin inhibited, "streptobacillus" associate.

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* Laboratory of Tropical Diseases.

The cultivation of *E. histolytica* with *Trypanosoma cruzi*[13, 22] followed considerable work by the author with the Shaffer-Frye cultures. During these studies, it was noted that any variation of the Shaffer-Frye technique which resulted in more rapid or complete inactivation of the streptobacillus was accompanied by either a decrease or a loss, respectively, of amoebic growth. If the concentration of penicillin was too great, or, if penicillin and streptomycin were employed through prolonged serial transfers, even in concentrations that were not amoebicidal, no growth of amoebae occurred. Furthermore, if the streptobacillus was completely inhibited by antibiotics in advance, then washed as free as possible of antibiotics, and added to the medium at the time of inoculation with amoebae, no growth of amoebae occurred. Other studies showed that filtrates or supernates of medium conditioned by growth of the streptobacillus failed to promote growth of *E. histolytica.* This failure of the amoebae to grow with the streptobacillus after treatment procedures that completely inhibited its metabolism or in conditioned medium from which it was removed suggested that the amoeba may be directly dependent upon the metabolism of associates. Perhaps the success of the Shaffer-Frye procedure was inherent in the employment of penicillin in a concentration adequate to inhibit multiplication of the bacterium while permitting some metabolic activity. Similar phenomena may have been operative in the heat-treatment procedures utilized by Jacobs.

The unfavorable outlook toward the cultivation of the amoeba in pure culture, resulting from the foregoing studies, stimulated the search for satisfactory amoeba-associates among the Trypanosomidae. *T. cruzi* was chosen because it was easily cultivated in a variety of media and, in contrast to the other species of Trypanosomidae, it was known to thrive at temperatures suitable to the amoeba. An inoculum of the F-22 strain of *E. histolytica* derived from 48-hour Shaffer-Frye cultures was transferred into Trypicase-serum-sodium thioglycollate medium which had received an inoculum of *T. cruzi.* Penicillin and streptomycin were added to ensure the complete inactivation of potentially carried-over streptobacillus. It was evident, after 24 hours' incubation, that *T. cruzi* possessed growth-promoting properties for the amoebae, since they grew at a rate comparable to that previously observed in the Shaffer-Frye cultures. This strain of *E. histolytica*, thus established with *T. cruzi*, is still being propagated after more than three years.

A total of nine strains of *E. histolytica* derived from other cultures or from cysts or trophozoites in fecal specimens has been isolated and maintained in amoeba-trypanosome medium.[14] There have been no failures in attempts to isolate various strains with *T. cruzi*, but there has been evidence for the existence of differences in the cultural requirements of various strains.

One strain, which was isolated from cysts in a specimen from an asymptomatic patient, was maintained for a period of six months during which time it failed to exhibit the rapid growth rate which characterized each of the other strains. When in association with its original flora, however, abundant growth of amoebae occurred.

Several strains of *T. cruzi* have shown no differences in their ability to promote growth of *E. histolytica*. However, similar studies with *T. conorhini*, *T. pipistrelli*, *Strigomonas fasciculata*, *Leishmania donovani*, *L. tropica*, and *L. brasiliensis* revealed that, although each species was ingested in large numbers by the amoeba, none was capable of promoting growth of amoebae through prolonged serial transfer in an environment wherein *T. cruzi* sufficed.[14] These data, together with those which show the inability of certain species of bacteria to promote growth of *E. histolytica*,[7, 15, 16] suggest a requirement by the amoeba for associates with certain as yet unidentified physiological characteristics.

The amoeba-trypanosome, A-T cultures, have provided inocula of bacteria-free amoebae for various other studies. Since *T. cruzi* survives only for a short time in L.E.R. medium, the problem of isolating the amoebae with designated bacterial associates has been simplified. Amoebae from A-T cultures may be transferred to L.E.R. medium containing desired bacterial associates. It is sometimes necessary, however, to inoculate a series of 8 to 10 culture tubes since, especially with certain bacteria, there is considerable mortality of the amoebae. Nakamura[17] has reported the failure of amoebae to grow when transferred from A-T cultures into medium with organism *t*, or the Shaffer-Frye streptobacillus and also, when transferred from association with either of these bacteria into medium with *T. cruzi*. In the present writer's hands, the same strains of amoebae as used by Nakamura, F-22, 103, and 200, have been transferred successfully under all of the above conditions of transfer. A heavy inoculum of bacteria, however, has usually been required. This might explain Nakamura's difficulty.

Studies of amoeba-trypanosome cultures and of the metabolism of *T. cruzi* have not as yet identified the factors contributed by associates for growth of *E. histolytica*. These investigations have shown that *T. cruzi*, heat treated at 48°C is capable of supporting some growth of the amoeba, but treatment at slightly higher temperatures destroys these growth-promoting properties.[22] Recent studies[23] show a direct correlation between complete suppression of the metabolism of the trypanosome and loss of these properties. Procedures which rendered *T. cruzi* immotile and incapable of multiplication, but still showing some metabolic activity, did not completely destroy the amoeba-growth-promoting properties. These studies suggest that the factors supplied by living microbial associates or other living cells are either very thermo-labile or intimately connected with the life of the cell.

The requirement mentioned earlier in the present paper for cultures of *E. histolytica* that may furnish material for serodiagnosis of amoebiasis has not thus far been met by A-T cultures. The possibilities of processing specific and potent antigens from this source, however, have not been adequately investigated. Some significant results on the screening of drugs *in vitro* for amoebicidal properties have been obtained with the use of A-T cultures.[19, 20, 21]

The demonstrated resistance of *T. cruzi* to the activities of many of the newer compounds renders it a suitable associate of the amoeba in studies of this kind. In the experiments of the writer[20] a microculture technique developed by Rees *et al.* was used, wherein the effects of drugs on single trophozoites were evaluated. The microculture method, by permitting direct observation of individual amoebas and the enumeration of their progeny, provides more specific information concerning the amoebicidal or amoebistatic activity of the drugs. In addition, the use of microtubes permits the testing of drugs available only in minute quantities.

The A-T cultures have also been employed in studies of the effects of cultural associates on the infectivity and pathogenicity of *E. histolytica* for experimental hosts. These studies indicate that the bacterial associates of the amoeba may participate in its establishment in the intestinal tract of experimental animals. In the work of Luttermoser and Phillips,[18] for example, only 1 of 24 rabbits that were inoculated with amoebae from A-T cultures became infected, whereas 17 of 34 animals inoculated with the same strain of *E. histolytica* growing with organism *t* became infected, 12 with fatal cases of amoebic dysentery.

Summary

Cultures of *E. histolytica-T. cruzi* without bacteria have been established and maintained for more than three years. All strains of the amoeba thus far investigated have been grown with the trypanosome. Six other species of Trypanosomidae have failed to exhibit amoeba-producing properties. By inoculation of amoebae from A-T cultures into medium seeded previously with selected species of bacteria it has been possible to establish any suitable amoeba-bacteria association. Some growth of *E. histolytica* has occurred in the presence of *T. cruzi* inactivated by heat or other agents so long as some residual metabolism in the trypanosome was demonstrable. Killed *T. cruzi* or products of its degradation have failed to show amoeba-producing properties. The data indicate that growth of *E. histolytica* may be dependent on the continuing metabolism of living microbial associates. Although the A-T cultures have not as yet provided suitable material for the processing of antigens for serodiagnostic tests of amoebiasis the cultures, especially microcultures, have been of value for the screening of potential amoebicides. Studies on infectivity and pathogenicity of *E. histolytica* in experimental animals, showing that amoeba-trypanosomes have little infectivity in comparison with certain amoeba-bacteria cultures indicate that bacteria may contribute toward the infectivity and pathogenicity of the amoeba.

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FACTORS AFFECTING THE PROPAGATION OF *ENDAMOEBA HISTOLYTICA IN VITRO* IN THE S-F MEDIUM AND IN TISSUE BEARING SUBSTRATE

By James G. Shaffer

Department of Microbiology and Public Health, The Chicago Medical School, Chicago, Illinois

In 1948, Shaffer, Ryden and Frye[1] reported a new medium for the propagation of *Endamoeba histolytica in vitro.* This medium, which hereafter will be referred to as the S-F medium, has proven quite useful in the study of certain factors affecting propagation of the amoebae. It lends itself to the application of quantitative procedures and has made possible some rather exacting determinations.

Recently, another technique for the propagation of *E. histolytica* has been developed in our laboratory, using chick embryo tissues suspended in tissue culture nutrient fluid. This medium has the great advantage of being free of bacteria, but the presence of chick embryo tissues introduces certain complications. Its possibilities appear to be very great.

This report will discuss only those observations made with the S-F or chick embryo culture media bearing on factors affecting the propagation of *E. histolytica.* Time and space do not allow for reference to all the valuable contributions of other workers on the cultivation of the intestinal amoebae. The presentation will be divided into three parts: the first devoted to certain physical factors and their effects on the propagation of the amoebae; the second to nutritional factors in the S-F medium; and the third to some preliminary observations on the chick embryo tissue bearing cultures.

Part 1. Physical Conditions

The effects of certain controllable physical conditions on the propagation of *E. histolytica* in the S-F medium have been investigated, including oxidation-reduction potential or anaerobiosis, pH, surface area and temperature.

Anaerobiosis. All workers are agreed that maintenance of low oxygen potential is essential to propagation of *E. histolytica in vitro.*[2, 3] With the S-F medium, it has been necessary to layer all cultures with vaseline to ensure exclusion of oxygen from the medium. Breaking the vaseline seal on cultures with large numbers of trophozoites present results in an accelerated death and disappearance of the amoebae. Thus, maintenance of anaerobiosis is essential not only for propagation, but also for survival of the amoebae. In a previous paper, the author[4] has reported on the effects of pH on the propagation of two strains of *E. histolytica* in the S-F medium. It was found that the pH of the medium could be adjusted to any desired level before inoculation with *E. histolytica* and that the pH would remain surprisingly constant throughout the period of observation.

The growth curves of *E. histolytica*, strain 200, in S-F medium of adjusted pH levels are shown in FIGURE 1.

FIGURE 1 reveals that pH 6.0 was optimal for propagation of *E. histolytica*, strain 200. Good propagation occurred over the range of pH 6.0 to 6.9, but was poor at pH 5.1, 5.4 and 7.4. Results with other strains have shown some variation but, in each case, the optimum pH was about 6.0.

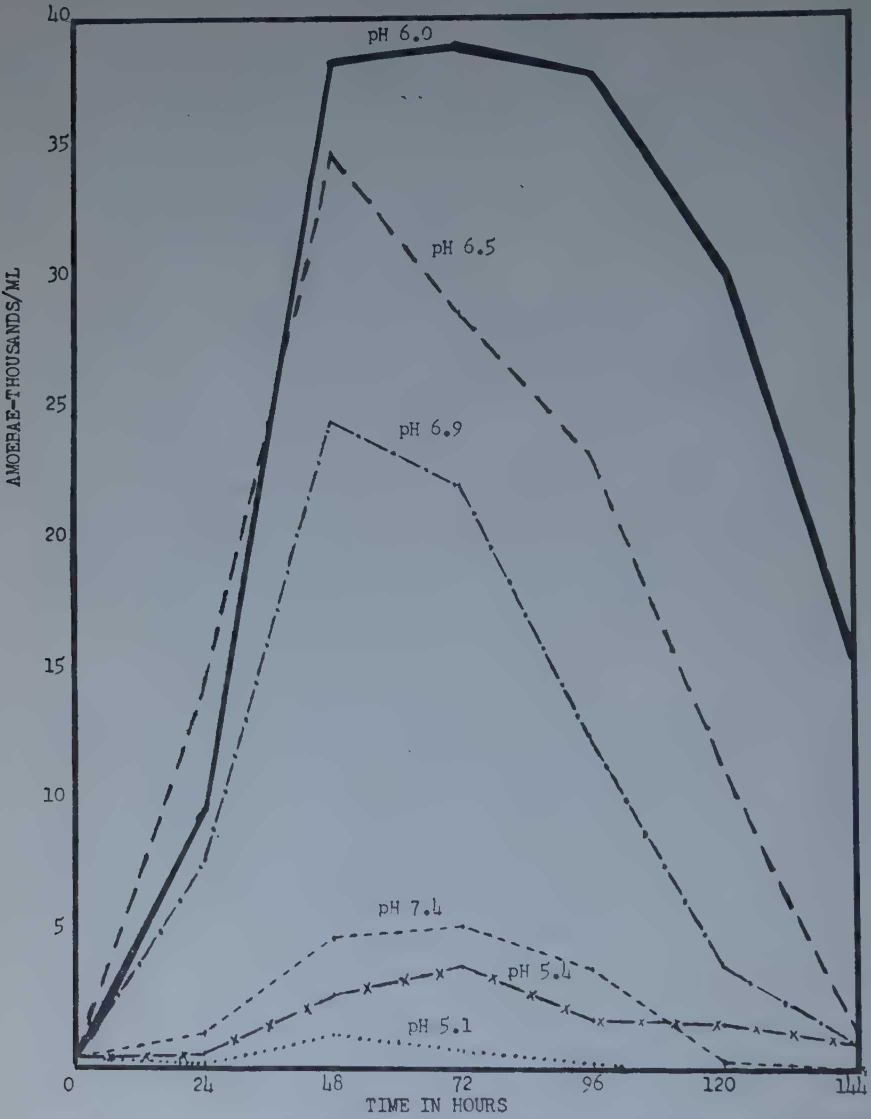

FIGURE 1. Growth curves of the 200 strain of *Endamoeba histolytica* in S-F medium at various pH levels.

The above results cannot be interpreted as establishing pH 6.0 as the optimal pH for *E. histolytica* under all circumstances, since excellent propagation of both the 200 and Luna strains occurs in tissue bearing substrate in which the initial pH is 7.6 with a reduction to pH 7.2 during 48 hrs. of incubation. The difference may be explainable according to the type of substrate available to the amoebae. Certain enzymes or other factors necessary for the amoebae and supplied by the different constituents of the medium may vary in the pH at which they are active. Thus, it is not certain whether it is the amoebae themselves that require a certain pH or whether the optimal pH for propagation is determined by the substrate.

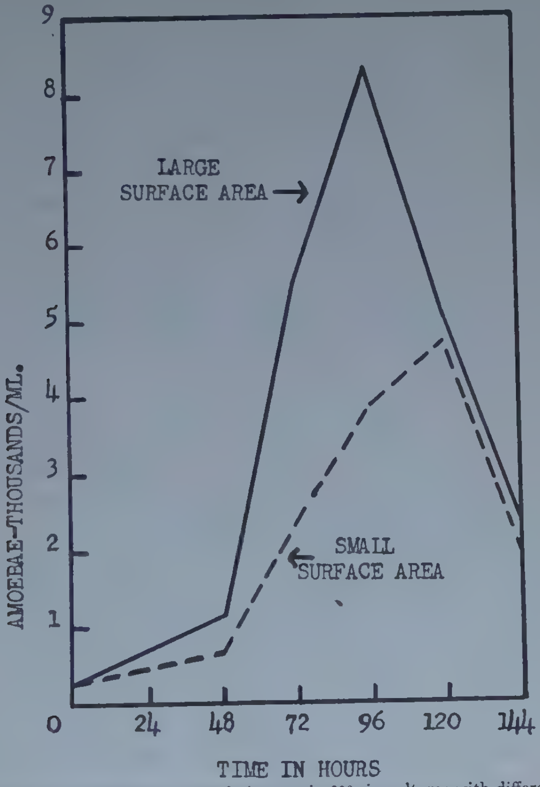

FIGURE 2. Growth curves of *Endamoeba histolytica*, strain 200, in cultures with different horizontal surface areas.

Surface area. The effect of the amount of horizontal surface area available in the cultures on the propagation of the amoebae has also been investigated. Similar amounts of medium were placed in each of a series of oval test tubes (23 × 11.5 × 152 mm.). A standard inoculum of *E. histolytica* (approximately 250 amoebae per ml. of medium) was then inoculated. Half of the cultures were incubated in a vertical position and the other half were incubated in a horizontal position with the flat side of the tube down, providing in one case a small horizontal surface area and in the other an area many times greater.

The growth curves of the amoebae cultured in the horizontal and vertical tubes are shown in FIGURE 2.

These growth curves reveal that the nature of the growth cycle was markedly altered by changes in the surface area. The peak of the growth cycle with a large surface area was reached between 72 and 96 hrs. and with the small surface area it was reached at about 120 hrs. A possible explanation of these results may be in terms of the depth of the medium which was obviously much less in the tubes lying flat than in tubes in a vertical position. Since other experiments have shown that the nutrient material in S-F medium is sedimentable and settles out on standing,[4] it may be that availability of nutrient material was responsible for the differences observed. So far, no experiment, in which equal volumes of medium with similar depth but varying surface are present, has been devised. Faust *et al.*[5] obtained similar results using Erlenmeyer flasks and conical test tubes, but made no statement as to the relative depth of the medium in the cultures. Their counts were all made after 72 hours' incubation.

Temperature. Little quantitative work has been done on the effect of variations in temperature. It is generally recognized that *E. histolytica* propagates best at temperatures of 37–37.5°C and the S-F medium is no exception.

To summarize, it has been found that oxidation-reduction potentials, pH, surface area, and temperature are important factors in determining the pattern of propagation of *E. histolytica* in S. F. medium. In all quantitative experiments it is essential to control these factors very closely.

Part 2. Nutritional Factors

Before considering the various nutritional factors which have been found important to the propagation of *E. histolytica*, it is necessary to outline the methods now used in making the S-F medium. TABLE 1 summarizes the method of preparation of the medium.

The supernatant fluid used as the base for the S-F medium, as shown in TABLE 1, contains 30 to 70 million viable streptobacilli per ml. The bacteria are sensitive to the penicillin G, and the concentrations used, as will be shown later, result in logarithmic death of the bacterial cells.

The factors in S-F medium responsible for the propagation of *E. histolytica* have been under investigation since its original development. It seemed quite likely at the outset that some conditioning process was being accomplished by the bacterial culture. Certain possible explanations were recognized: (a) The bacteria act upon the fluid thioglycollate medium directly, producing some soluble metabolic product or products, or some set of physical conditions essential to the amoebae, (b) the bacterial cell or some part of it is essential to the metabolism of the amoebae, or (c) some combination of (a) and (b) may be accomplished in the conditioning process.

In TABLE 2 are listed the constituents of the fluid thioglycollate medium used to grow the streptobacillus in preparation for making the S-F medium alongside the constituents of the S-F medium itself.

Of the constituents of the fluid thioglycollate medium, those investigated to date include the peptone (trypticase), the carbohydrate (dextrose), methylene blue, and rice powder.

Peptone. It was surprising to find that of seven commonly used peptones, none could be substituted for the trypticase in the basic fluid thioglycollate medium. Although some of them supported excellent propagation of the gram-negative streptobacillus, in no case did the S-F medium, made up using supernatant fluids from these cultures, support propagation of *E. histolytica*. Thus, the type of peptone used in the fluid thioglycollate medium is very important and, so far, no substitute for trypticase has been found.

Methylene blue. With regard to the methylene blue, it has been found that it can be omitted or its concentration can be greatly increased with no notable effect on the propagation of either the streptobacillus or the amoebae.

Rice powder. Rice powder has been used routinely, as indicated in TABLE 2. However, experiments have shown that it can be omitted with little effect on the propagation of the amoebae in the S-F medium. The only justification for its routine use is the general observation that more consistent results are obtained on routine transplants in the maintenance of the *E. histolytica* cultures in the laboratory. It can be safely assumed that the rice powder provides nothing absolutely essential to the propagation of *E. histolytica* in the S-F medium.

TABLE 1

METHOD OF PREPARATION OF THE S-F MEDIUM

(1) 24 hr. culture of unidentified gram negative anaerobic streptobacillus (bacteroides sp ?) in Baltimore Biological Laboratory's Fluid Thioglycollate Medium #136 with 1.0 per cent dextrose and 1.0 per cent rice powder added.

(2) Centrifuge at 2000 R.P.M. (International #2 centrifuge) under vaseline seal for 45 minutes.

(3) Collect supernatant fluid and discard sediment.

(4) Make medium: 2.5 ml. supernatant fluid, 0.1 ml. normal horse serum, 1000 units penicillin G (in 0.7 ml. 0.85 per cent NaCl).

TABLE 2

CONSTITUENTS OF THE FLUID THIOGLYCOLLATE MEDIUM AND OF THE S-F MEDIUM

Fluid Thioglycollate Medium (Formula In Grams Per Liter)		S-F Medium (Constituents of a Single Tube Culture) (Ml.)	
Trypticase	20.0	Supernatant fluid (Contains bacterial cells, unchanged constituents of the fluid thioglycollate medium and products of bacterial metabolism).	2.5
Sodium chloride	5.0		
Dipotassium phosphate	2.0		
Sodium thioglycollate	1.0		
Methylene blue	0.002		
Agar	0.5	Normal horse serum	0.1
Dextrose*	10.0	Penicillin G, potassium (1000 u)	0.7
Rice powder†	—		

* Dextrose is added to the dissolved medium just prior to autoclaving at 15 lbs. pressure for 10 min.

† Rice powder is added to the complete medium at the time it is inoculated with the streptobacillus (approximately 1.0 per cent).

Carbohydrate. Experiments reported by Ryden,[6] who used a somewhat modified medium utilizing the streptobacillus without penicillin as originally described by Shaffer, Walton and Frye,[7] showed that only those carbohydrates which might be classed as being related to dextrose; that is, maltose, dextrin and glycogen, could be used in the medium for the propagation of *E. histolytica*. His results did not answer the question as to whether the carbohydrate was being utilized by the streptobacillus or the amoebae, or by both organisms. He found that the streptobacillus fermented certain other monosaccharides, but none other than those mentioned above supported propagation of *E. histolytica.*

Since Ryden found that dextrose and maltose were equally effective in his experiments and that dextrin and glycogen were less active in that order, it became of interest to investigate quantitatively the relationship of dextrose and maltose to the propagation of *E. histolytica* in the S-F medium.

Preliminary experiments showed that, if the streptobacillus were cultured for 24 hrs. in fluid thioglycollate medium containing only 0.1 per cent dextrose, the propagation of the bacteria was comparable and the resulting supernatant fluids had viable bacteria similar in number to those prepared from cultures in medium containing 1.0 per cent dextrose. The number of bacteria present in the supernatant fluid is of great importance as will be indicated later.

It was found, however, that when supernatant fluids prepared from streptobacillus cultures grown in fluid thioglycollate medium containing 0.1 per cent dextrose were used in making S-F medium, no propagation of *E. histolytica* occurred. This observation made possible the study of the effect of adding varying concentrations of dextrose and maltose to S-F medium.

The following experiment was done to study the relationship between the concentration of dextrose and maltose and the ability of *E. histolytica* to propagate in S-F medium.

Supernatant fluid prepared from 24 hr. cultures of the streptobacillus in fluid thioglycollate medium containing 0.1 per cent dextrose was used to prepare two series of tubes of S-F medium. To one series were added increasing amounts of dextrose in saline (0.85 per cent NaCl), and to the other series were added similar amounts of maltose. All tubes were inoculated with a standard inoculum of 280 washed amoebae per ml. of medium. The cultures were then incubated at 37°C for 96 hrs. and the amount of propagation determined by counting the amoebae in each culture. The technique for doing these counts has been reported elsewhere.[4]

The results of the experiment using varying concentrations of dextrose and maltose are shown in TABLE 3.

It will be noted from the results shown in TABLE 3 that, as the concentrations of each carbohydrate decreased, there was a decrease in the propagation of the amoebae. These results, along with those to be presented later, are interpreted as presenting strong evidence that dextrose or maltose are metabolically related to the propagation of *E. histolytica* in S-F medium. Thus, it would appear that the function of the carbohydrate in the S-F medium is two-fold; that is, the carbohydrate is important in culturing the streptobacillus in preparation for obtaining the supernatant fluid and also functions during the propagation of the amoebae. As will be shown later, however, other carbohydrates can be substituted for the dextrose or maltose in culturing the streptobacillus.

The influence of the more important constituents of the S-F medium itself have been studied quantitatively, most of the work being involved with the supernatant fluid which actually constitutes the base for the medium. Experiments presented elsewhere[4] have shown that the soluble products of the bacterial metabolism in the fluid thioglycollate medium are not essential to the amoebae. Quite to the contrary, the necessary growth factor or factors reside either in or upon the bacterial cells or some similar particulate substance contained in the supernatant fluid used to make up the medium. It has been shown that there exists a direct relationship between the number of viable bacterial cells in the supernatant fluid and the amount of propagation of the amoebae.

The experiment to be described next was designed to study further the relationships between bacteria and amoebae in the S-F medium, and to determine whether or not bacterial cells grown in the presence of a carbohydrate other than dextrose or maltose could be used.

TABLE 3

RELATIONSHIP OF DEXTROSE AND MALTOSE TO PROPAGATION OF *E. Histolytica* (STRAIN 200) IN S-F MEDIUM

Concentration* of Carbohydrate in Medium (Mg./Ml.)	Amoebae† No./Ml. at 96 Hrs.	
	Dextrose	Maltose
5.4800	18,437	14,078
1.3700	10,960	10,632
0.3400	8,132	14,703
0.0850	7,820	10,005
0.0210	4,060	1,618
0.0050	648	2,187
0.0010	313	234
0.0002	156	—
0	156	78

* The concentration indicated in this column is that concentration resulting from the addition of dextrose or maltose. Actually, the concentration of dextrose is 0.5 mg./ml. higher than the figures show since that amount of unused dextrose remains in the supernatant fluid.

† The inoculum was 280 washed amoebae per ml.

The monosaccharide, D-galactose was chosen as the carbohydrate to replace dextrose in the fluid thioglycollate medium since Ryden[6] had found the streptobacillus was capable of fermenting this sugar.

Twenty-four hour cultures of the streptobacillus in fluid thioglycollate medium containing 1.0 per cent d-galactose and 1.0 per cent dextrose were centrifuged at low speed for 30 min. to remove all gross particles. The supernatant fluids from this initial centrifugation were then re-centrifuged at 3000 r.p.m. in the International #2 centrifuge for 45 minutes. In this manner, four bacterial sediments were prepared, two contained streptobacilli grown in medium containing dextrose and the other two contained streptobacilli grown in medium containing D-galactose. The supernatant fluid was completely removed from the tightly packed bacterial sediments and a portion of each supernatant used to make S-F medium. Simultaneously, viable streptobacillus counts were done on each supernatant fluid.

Suspensions of the streptobacilli in the four tubes were then made and treated as shown in TABLE 4. The results of this experiment are shown in TABLE 5.

TABLE 4

METHOD OF DOING EXPERIMENT UTILIZING SUSPENSIONS OF THE GRAM NEGATIVE STREPTOBACILLUS GROWN IN FLUID THIOGLYCOLLATE CONTAINING DEXTROSE OR D-GALACTOSE

Bacteria grown in FTg containing	Dextrose		D-Galactose	
Sediment	1	2	3	4
Suspend in 3.0 ml. FTG* pH 6.1 containing 1% carbohydrate	Dextrose	D-galactose	Dextrose	D-galactose
Procedure for doing experiment	Make count to determine number of viable streptobacilli in each suspension.			
	Make serial 2-fold dilutions (1:1 to 1:32) of each bacterial suspension using FTG*, pH 6.1 containing 1.0% of carbohydrate corresponding to that in suspending FTG indicated under 1, 2, 3, and 4 above.			
	Add 0.5 ml. of each of the dilutions to appropriate tube made up with 2.0 ml. FTG, pH 6.1 containing 1.0% of the carbohydrate corresponding to that used in making the bacterial suspension, 0.1 ml. of normal horse serum and 0.7 ml. of saline containing 1000 units of penicillin G.			
	Inoculate with 250 amoebae per ml. of inoculated medium and incubate at 37°C. for 72 hrs.			
	Count amoebae in each tube.			

* Fluid Thioglycollate Medium (Baltimore Biological Laboratories #136).

TABLE 5

RESULTS OF EXPERIMENT USING BACTERIAL CELLS (STREPTOBACILLI) CULTURED IN DEXTROSE OR D-GALACTOSE CONTAINING MEDIUM AND ADDED IN VARYING NUMBER TO MODIFIED S-FMEDIUM USING EITHER DEXTROSE OF D-GALACTOSE AS CARBOHYDRATE

Tube	Series							
	1		2		3		4	
	Bacterial Cells From Dextrose Medium Suspended in Dextrose Medium		Bacterial Cells From Dextrose Medium Suspended in D-Galactose Medium		Bacterial Cells From D-Galactose Medium Suspended In Dextrose Medium		Bacterial Cells From D-Galactose Medium Suspended in D-Galactose Medium	
	Amoebae* Per Ml. (72 Hr.)	Bacteria Added (Millions/Ml.)	Amoebae Per Ml. (72 Hr.)	Bacteria Added (Millions/Ml.)	Amoebae Per Ml. (72 hr.)	Bacteria Added (Millions/Ml.)	Amoebae Per Ml. (72 Hr.,	Bacteria Added (Millions/Ml.)
1	65,469	35.8	0	52.4	48,828	78.1	0	62.5
2	41,172	17.9	0	26.2	23,828	39.0	0	31.2
3	18,750	8.9	0	13.1	2,500	19.5	0	15.6
4	7,422	4.8	0	6.5	156	9.7	0	7.8

* The 200 strain of *E. histolytica* was used in this experiment.

Note: Supernatant fluid from dextrose culture—5.5 million bacteria/ml.—had 703 amoebae/ml. after 72 hours. Supernatant fluid from d-galactose culture—2.9 million bacteria/ml.—had 0 amoebae/ml. after 72 hours.

Examination of the results shown in TABLE 5 reveals the following facts: Bacterial cells grown in the presence of either dextrose or D-galactose and added to fluid thioglycollate medium containing dextrose supported propagation of *E. histolytica* (Series 1 and 3), whereas similar bacterial cells added to fluid thioglycollate medium containing D-galactose failed to support propagation of *E. histolytica* (Series 2 and 4). The results shown in Series 1 and 3 may indicate that bacteria grown in D-galactose medium were not so active as and, therefore, may have differed qualitatively from, those grown in dextrose in supporting propagation of the amoebae, since 78.1 million D-galactose bacteria yielded approximately the same number of amoebae as 17.9 million dextrose bacteria. Series 1 and 3 show that a direct quantitative relationship exists between the number of bacterial cells in the medium and the propagation of the amoebae. Further confirmation that soluble metabolites present in the supernatant fluid are not important for the propagation of *E. histolytica* can be seen in the results obtained using the dextrose supernatant fluid as indicated by the note at the bottom of the table. This supernatant fluid contained 5.5 million bacteria, a number similar to the 4.8 million bacteria in tube 4, Series 1, but the propagation of the amoebae in the supernatant fluid medium was even less than that in the tubes with fresh fluid thioglycollate medium to which the bacterial cells were added.

Thus, the results shown in TABLE 5 tend to confirm the importance of the bacterial cells and to further rule out soluble metabolic products, in the supernatant fluid used to make S-F medium, as growth factors for *E. histolytica.* They further emphasize the importance of dextrose. As mentioned previously, other evidence has indicated that *E. histolytica* utilizes dextrose in its metabolism. There is also the possibility that some labile metabolite was being produced by the bacterial cells, even though their propagation was evidently completely stopped by the penicillin G in the medium, as will be shown later. This seems unlikely, since, if it were true, the supernatant fluid should support propagation of the amoebae equally well with similar numbers of bacterial cells, as does the fluid thioglycollate medium containing the resuspended bacteria. The results shown in TABLE 5 do not indicate that this occurred.

Penicillin G. The role of penicillin G in the S-F medium appears to be mainly concerned with the bacteria present in the supernatant fluid, although omission of the antibiotic has a definite effect on the growth curve of the amoebae, as shown by the following experiment.

Four series of tubes of S-F medium were set up. The first series was made in the usual manner and inoculated with a standard dose (250 amoebae/ml.) of the 200 strain of *E. histolytica* trophozoites suspended in fluid thioglycollate medium. The second series was not inoculated with amoebae, but received fluid thioglycollate medium to equal the volume of the inoculum used in the first series. The third and fourth series were identical with the first and second, except that penicillin G was omitted, 0.7 ml. of saline being substituted to maintain volumes similar to the first two series.

All tubes were layered with vaseline and incubated at 37°C. Beginning at 0 hrs. and at 24 hr. intervals, thereafter, amoeba and viable streptobacillus counts were done on tubes from Series 1 and 3 and bacterial counts were done on Series 2 and 4 which had no amoebae.

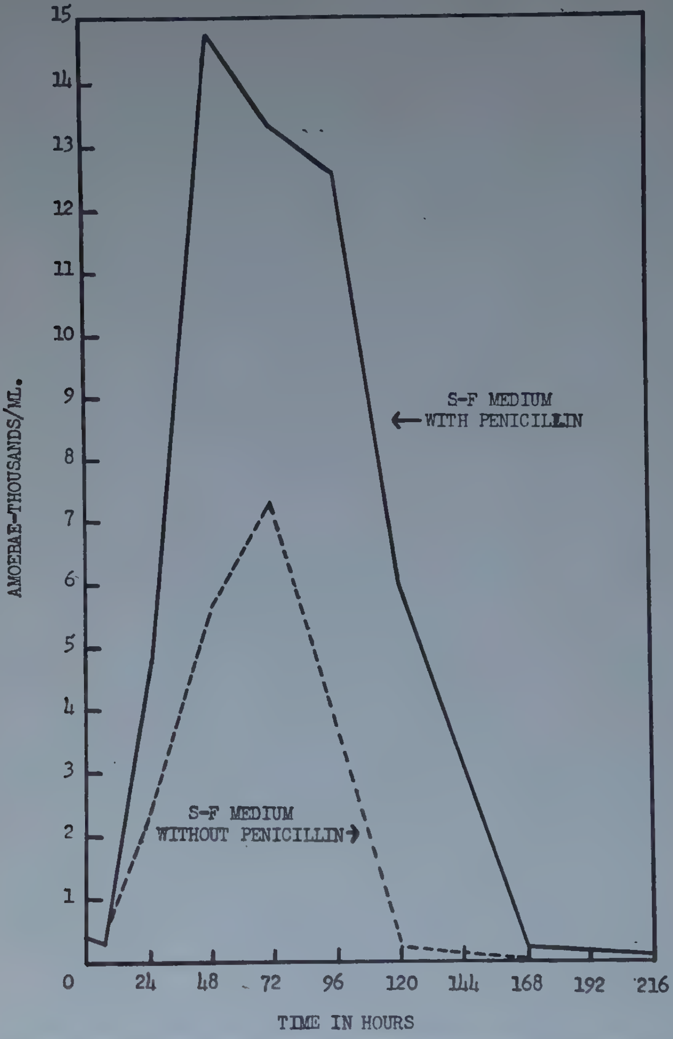

FIGURE 3. Growth curve of the F-22 strain of *Endamoeba histolytica* in S-F medium. Experiment #2. (Arithmetic scale)

The results of these experiments are shown in FIGURES 3 and 4.

In FIGURE 3 is shown the growth curves of the 200 strain of *E. histolytica* in S-F medium with and without penicillin G. It will be seen that omission of the antibiotic results in much less propagation of the amoebae. This is thought to be due to changes in pH that occur in the medium without penicillin G. The pH under these circumstances changes from the original 6.1 to a pH of 5.5 or

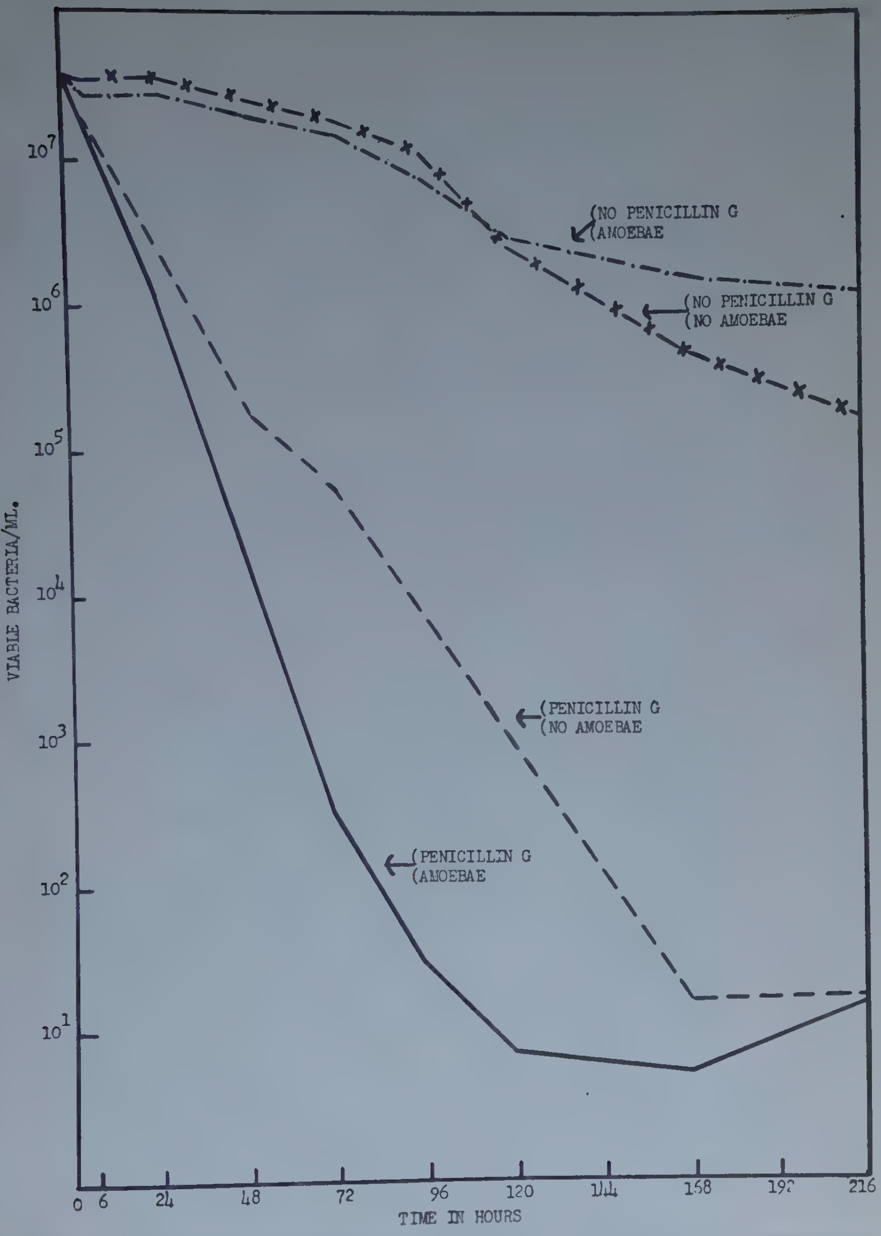

FIGURE 4. Death curves of the streptobacillus in S-F medium with and without penicillin G. (Semi-logarithmic scale)

5.6 over the first 48–72 hrs. of incubation. The amoebae, as shown previously, do not propagate well at the lower pH.

FIGURE 4 shows the survival curves of the streptobacillus in the four series of tubes of S-F medium. It will be seen that (a) the bacteria go into a logarithmic death phase, as measured by their ability to propagate when placed in a suitable substrate, immediately on coming in contact with the penicillin G, (b) the slope of the death curve is greater in the presence of propagating *E. histolytica*, (c) in the absence of penicillin G the number of viable streptobacilli remains surprisingly constant for 48–72 hrs. and then decreases over the next few days, (d) little difference was observed with or without propagating amoebae in the absence of penicillin G.

Thus, penicillin G exerts a profound effect on the viable bacterial cells in the S-F medium and its function in the cultures can probably be considered referable to this action. The presence of propagating amoebae appears to increase the rate of death of the bacterial cells. It is not certain whether this indicates bactericidal action on the part of the amoebae or that the amoebae are competing with the viable bacteria for some vital substance not capable of being reproduced by the bacteria.

TABLE 6

RELATION OF PROPAGATION OF *Endamoeba histolytica*, STRAIN 200, TO THE CONCENTRATION OF NORMAL HORSE SERUM IN S-F MEDIUM

Concentration of Normal Horse Serum (Per Cent)	Number of Amoebae* Per Ml. After 72 Hrs. At 37 C
0	0
0.02	0
0.04	156
0.22	4,492
0.44	5,625
2.22	7,422
4.44	9,531
8.88	9,219
14.62	7,403
22.22	5,703

* Original inoculum—250 amoebae per ml. of medium.

Normal horse serum. As previously indicated, it has been found that normal horse serum is necessary for propagation of *E. histolytica* in S-F medium although rabbit or human serum can be substituted for it. The experiment to be described next was designed to study quantitative relationships between the concentration of normal horse serum and propagation of *E. histolytica.*

Tubes of S-F medium were set up in the usual manner, omitting the normal horse serum. Varying quantities of the serum were then added to the tubes in a volume of 1.0 ml., using saline as a diluent. Careful attention was given to keeping the pH of these tubes constant at 6.1. The tubes were then inoculated with a standard inoculum (250 amoebae per ml.) of washed amoebae. After layering with vaseline, the cultures were incubated at 37°C for 72 hrs. and the number of amoebae per ml. determined.

The results of this experiment are shown in TABLE 6.

It is apparent that there was a definite relationship between the concentration of normal horse serum in S-F medium and the propagation of *E. histolytica.* The optimal range of concentration appeared to be from about 2 to 9 per cent. With 14.62 and 22.22 per cent horse serum propagation of the amoebae was somewhat inhibited. Other experiments have shown that this inhibitory action of the normal horse serum begins at concentrations of about 10 to 12 per cent. No experiments have been attempted using serum fractions.

It is not known what the function of the horse serum is in the medium. Three possibilities are recognized, (a) it may provide nutrient material for the amoebae, (b) it may neutralize some toxic material in the medium or (c) it may combine both of the above properties. The true nature of its activity awaits further investigation.

Part 3. Tissue-Bearing Cultures

As mentioned previously, another culture medium for the propagation of *E. histolytica* has recently been developed which utilizes tissues obtained from chick embryos suspended in tissue culture nutrient fluid.[8] This medium appears to be free from all viable microorganisms. A more detailed account of the findings with the tissue-bearing cultures is being published elsewhere and only a preliminary account will be given here as it bears on the discussion of nutritional factors concerned in the propagation of *E. histolytica.*

It has been found that tissues of chick embryos of from 4 to 14 days' incubation when ground in a mortar without abrasive and suspended in 10 per cent concentration in tissue culture nutrient fluid support good growth of *E. histolytica.* This was true if the cultures were incubated either in cotton-plugged tubes in the anaerobe jar or aerobically in rubber stoppered test tubes. Brain, liver, muscle, heart and intestinal tissues have been found capable of supporting propagation. Therefore, all experimental work has been done using the whole embryo.

The growth curve of the amoebae in the tissue-bearing cultures is quite similar to that in the S-F medium, maximum numbers (4,000 to 6,000 amoebae per ml.) being reached after 48 to 72 hours of incubation at 37°C. Experiments have shown that a 10 per cent suspension of the tissues yields the best growth of the amoebae, there being a definite correlation between tissue concentration and propagation as the per cent is decreased. With increases above 10 per cent, propagation of the amoebae also becomes less, possible due to the greater pH change.

Certain factors have been studied with regard to their effect on the multiplication of *E. histolytica* in the tissue-bearing cultures. First, it has been found that the presence of tissue cells is essential in the medium. Cell-free extracts have failed to support propagation of the amoebae. Second, the concentration of normal horse serum in the nutrient fluid affects the propagation, but it is not certain whether the primary effect is on the tissues or on the amoebae. Third, the pH of the medium is about 7.5 to 7.6 when made up and inoculated with amoebae and goes down to 7.1 to 7.2 during the 48 to 72 hours' incubation at 37°C. This differs markedly from the pH of the S-F medium which is ordinarily 5.9 to 6.2.

Experiments have shown that propagation of the amoebae is better if inoculation is made immediately rather than after the tissue-bearing substrate has been incubated at 37 C for 24 hours. This is interpreted as indicating that the constituents of fresh tissue cells are more important than any by-products of the cellular metabolism that might be in the medium after incubation.

Thus, the tissue-bearing substrate presents a medium in which the conditions for propagation of *E. histolytica* are in some ways quite different from those existing in the S-F medium. No bacterial cells, fungi or other protozoa are present in the cultures. The pH is considerably higher and, of course, the constituents of the medium are quite different from those of S-F medium. The tissue cells are apparently capable of replacing the bacterial cells present in the S-F substrate, but whether or not their contribution is the same still remains to be elucidated.

Discussion and Summary

The ultimate goal of all *in vitro* experiments on *Endamoeba histolytica* of the type just described is the propagation of the parasite in pure culture. It is of interest, therefore, to speculate on the properties and constituents of a medium which might be itself capable of supporting propagation of *E. histolytica*, *in vitro*. In TABLE 7, an attempt has been made to summarize the various conditions and constituents which might go into a medium for the propagation of *E. histolytica*. Certain things such as Na thioglycollate and agar have been omitted from the table since present evidence indicates that they are of less importance to the amoebae than to the streptobacillus in the initial phase.

TABLE 7
A HYPOTHETICAL MEDIUM FOR THE *in Vitro* PROPAGATION OF *E. Histolytica*

Conditions	Constituents
1. *Anaerobiosis*	1. Peptone (trypticase)
2. *pH* (apparently variable depending on the substrate)	2. Dextrose or maltose (1 per cent)
3. *Temperature* (37°C.)	3. Normal horse serum (2 to 9 per cent)
4. *Horizontal surface area*	4. Bacterial cells (streptobacillus-bacteroides, sp. ?)

Of the constituents shown in TABLE 7, all have been shown to be essential for the propagation of *E. histolytica*, *in vitro*, in S-F medium. The fundamental contribution of no one constituent is completely known at this time. It is to be suspected, however, that an elucidation of what may be termed the "missing link" supplied by the bacterial cells may supply information as to the true function of the other constituents. It would appear that the next line of approach should involve bio-chemical and physiological investigation of the bacterial cells.

On the basis of results obtained to date and, by analogy, it would appear that in the cultures with chick embryo tissues, the tissue cells hold the key to the propagation of *E. histolytica*. Further investigations on the tissue cells are thus indicated.

In the past, it has been the general opinion that the streptobacillus in the case of the S-F medium and the bacterial flora in certain other commonly used media, in some way condition the medium for propagation of *E. histolytica.* In the light of the observations herein reported, it seems likely that the conditioning hypothesis is valid only for production of anaerobiosis and proper pH, properties that can be controlled without the aid of the bacterial cultures. Conditioning in the sense of the production of metabolic products by alteration of constituents of the medium appears to be unimportant in the S-F medium and it may be unimportant in other media as well. Thus, the critical function of the bacterial growth appears to be the production of sufficient proper bacterial cells to provide the situation necessary for the amoebae to propagate. If these bacteria are "conditioning" the medium, it is a very short term process, with little or no carry-over in their absence. This latter possibility seems unlikely in the light of certain other evidence.[4]

In summary, the effect of certain of the more important physical and nutritional factors in the S-F medium and in tissue bearing substrate on the propagation of *E. histolytica* have been investigated and discussed. A tentative formula for producing a medium for the propagation of *E. histolytica* in pure culture has been given. A brief description of a new medium which is free of bacteria and its differences and similarities to the S-F medium has been made.

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IN VITRO GROWTH AND METABOLISM OF *ENDAMOEBA HISTOLYTICA*

By Quentin M. Geiman and Charles E. Becker
Department of Tropical Public Health, Harvard School of Public Health, Boston, Mass.

Introduction. Precise studies on the *in vitro* growth and metabolism of *Endamoeba histolytica* have been hampered by the inability to grow this disease-producing parasite without bacteria. Numerous investigators have attempted to discover and define the growth and metabolic characteristics of *E. histolytica* since the organism was first cultivated by Boeck and Drbohlav in 1925, but no one has succeeded in obtaining *in vitro* growth and multiplication in a completely-defined medium without bacteria or bacterial products, rice starch or flour, or *Trypanosoma cruzi* for long term experiments. Thorough reviews of the results of these investigations have been published in recent years by Anderson and Hansen (1947), Chang (1948), Deschiens (1950) and Frye (1951). No attempt will be made in this paper to bring these reviews up to date. Instead, we wish to present our approach to the problem undertaken a little over one year ago. This work was initiated at the request of the Commission on Enteric Infections of the Armed Forces Epidemiological Board with the hope that the methods previously developed for the *in vitro* cultivation of malarial parasites (Geiman *et al.*, 1946) might be adapted to similar studies on the chemical composition and metabolism of *E. histolytica.*

Synergism between *E. histolytica* and bacterial associates and its highly probable relationship to pathogenicity is a kind of paradox without parallel among the other parasitic protozoa. Why does *E. histolytica* invade the tissues and produce disease in one individual and merely inhabit the lumen of the large intestine in another individual with a different intestinal flora? Experimental evidence from animal inoculations (Spector, 1935, Deschiens, 1938) suggested that certain species of bacteria have a primary role in setting the stage for tissue invasion by the amoebae. The inability of Cleveland and Sanders (1930) and Snyder and Meleney (1940) to get growth of the amoebae after excystation in a bacteria-free medium shows that bacteria are needed, either to condition the medium physically or to produce metabolic products needed by the amoebae for growth and multiplication. The work of Rees and his collaborators (Rees *et al.*, 1944), over a period of years at the National Institutes of Health, and of Balamuth and Wieboldt (1951) has repeatedly emphasized the importance of the bacterial flora to the growth and metabolism of *E. histolytica.* The demonstration in recent years that antibiotics, including aureomycin, terramycin, bacitracin, and fumagillin, lead to clinical cure of a high percentage of individuals carrying *E. histolytica* provided us with new tools to treat the infection and for research purposes. When *in vitro* studies with these antibiotics, except for fumagillin (McCowen *et al.*, 1951), showed little direct effect on the amoebae, the primary role of bacterial associates in the growth and multiplication and, perhaps, pathogenicity of *E. histolytica* became apparent once again.

In considering the method of approach that might be used to hasten the accumulation of reproducible data about the nutrition and metabolism of *E. histolytica*, we decided to grow quantities of *E. histolytica* by the best method available for chemical analysis and metabolic studies. We can wait no longer for the discovery of a satisfactory method to grow *E. histolytica* free of bacteria for biochemical studies. We believe that methods are available from the fields of bacteriology, virology, and hematology to separate *E. histolytica* from bacterial associates and starch and to give a suspension of sufficient purity so that a toe hold can be gained in finding out something about the chemical composition and metabolism of this organism. We fully realize the many pitfalls and difficulties in this type of approach, but if the physical chemists can separate leucocytes and platelets from whole blood for chemical study, if biochemists can produce relatively pure suspensions of liver fractions or nuclear fractions from cells for chemical study, we should be able to achieve some measure of success with an initial suspension containing amoebae and bacteria. These two types of organisms have diverse physiological properties, and their physical dimensions and shape differ sufficiently to warrant attempts at separation by physical methods.

To obtain some idea of the quantity of amoebae needed for separation and biochemical studies, calculations have been made to approximate the volume of one amoeba in order to determine the number of the organisms and, hence, cultures needed to produce the necessary concentrates. If the average trophozoite is assumed to be spherical and to have a diameter of 20 microns, one amoeba would have a volume of 4.189×10^{-9} cm.^{3}, requiring approximately 238,000,000 amoebae to give 1 cm.^{3} of packed organisms, or, with an approximate density of 1, the average trophozoite would then weigh 4.189×10^{-6} mg. With the assumption that approximately 80 per cent of a trophozoite is water, the average trophozoite contains 8.4×10^{-7} mg. of solid matter. Thus, 10,000,000 trophozoites should yield approximately 8.4 mg. of solid matter, a sufficient quantity for microchemical analysis and studies. Approximately one-third more cysts would be needed for the same volume of material because of their smaller size. The density of cytoplasm in the cysts, however, is so much greater than that of trophozoites that volume differences might be ignored. Thus, our attention for some months has been concentrated on the mass production of amoebae for separation and chemical studies. We can produce the required amount of trophozoites by methods to be described below but we have not been able, as yet, to produce large numbers of cysts consistently.

Choice of strain for study. In going over the extensive literature on the cultivation of *E. histolytica*, there are not only a wide range of media available for use and an extraordinary variety of strains, but also very diverse results have been reported by different investigators using the same strain and, supposedly, the same medium for cultivation.

From previous experience with malarial parasites, we believe that it is necessary to conduct the studies with a known, recently-isolated pathogenic strain of *E. histolytica*, a strain that reproduces well in culture, and one that can be made to produce cysts *in vitro* at will. Even though extensive work has been done with the classical NRS strain of Dobell, this strain was rejected because of its origin from monkeys. Since considerable work has been done with the DKB strain, which is of human origin, this strain was obtained from Doctor J. N. DeLamater of the University of California for comparative studies. A more recently-isolated pathogenic strain was then sought for the bulk of our experiments. Fortunately, the Commission on Enteric Infections of the Armed Forces Epidemiological Board has organized a team to investigate amoebic dysentery on the island of Koje-do among North Korean prisoners of war, during the summer of 1951. In this study, 14 strains of *E. histolytica* were isolated (W. W. Frye, 1951), and some of these strains were brought back to this country. Through the courtesy of Doctor William Frye and Doctor Marion Brooke, who were members of the team, two strains designated as HK-9 and HK-14 were obtained. These two strains were tested in Boston for their experimental suitability with respect to cultural characteristics and pathogenicity for hamsters (method of Reinertson and Thompson, 1951). The HK-9 strain proved to be the most prolific in growth and pathogenicity for hamsters, so that it has been used for the bulk of our experiments. More recently, another strain was brought back (Q.M.G.) from Tokyo, Japan (courtesy of Col. R. A. Radke, Tokyo Army Hospital), for inclusion in our studies. This strain originated from a patient who developed his initial disease in Indo-China and it has proven to be even more pathogenic for hamsters than the HK-9 strain.

Before passing on to the mass production of trophozoites of amoebae, we wish to point out the need for a standardization of strains used for experimental studies with *E. histolytica.* The Subcommittee on Amebiasis of the Commission on Enteric Infections, A.F.E.B., has taken a step in this direction by agreeing to use the DKB and HK-9 strains for basic studies, and also this Subcommittee has sought a method for designating strains. A tentative method was adopted for trial by which the letters H or M would be used to signify a strain of human or monkey origin with the addition of the first letter of the name of the country from which the strain originated. For example, HK-9 means that the strain is of human origin and the ninth strain isolated in Korea. The strain from Indo-China has been designated as the HI-1 strain.

It is realized that the attempt to standardize strains of *E. histolytica* for experimental purposes is not without some disadvantages just as long as the organisms have to be grown in the presence of bacteria. Investigators might obtain the HK-9 strain for study but very soon find that the bacterial associates have changed from those in the original culture. If genetics means anything, however, the amoebae are still line descendants from the initial inoculum and presumably with the same disease potential when associated with the right species of bacteria. At least, we can make an attempt to standardize the strains, so that there is a basis for the confirmation of work done by different investigators. Whether or not this suggestion of a small number of investigators is feasible or practical will depend upon the wishes of other workers and the test of time. Strain designation of avian malarial parasites for research purposes was agreed upon some years ago by investigators with mutual benefit for all and a similar agreement in our opinion would benefit investigators working with *E. histolytica*.

Cultivation. The classical methods of cultivating *E. histolytica* in standard-size bacteriological tubes or flasks were not considered suitable for initial mass production of amoebae because of the inability to control the physiological environment for a sufficient length of time and because of harvesting difficulties. These objections applied also to cultivation of the amoebae by the rocker-dilution method originally devised in our laboratory for the cultivation of malarial parasites. For superior results, we have modified the rocker-perfusion boats used for the cultivation of plasmodia (Geiman *et al.*, 1946) by changing the shape of the vessel and increasing the diameter of the bell on which a cellophane membrane is tied (FIGURE 1). The present type of perfusion jar (PJ) gives a two-phase system with amoebae growing on a larger membrane in the bell and with the membrane touching a reservoir of nutrient solution in the bottom of the jar. This PJ vessel for the cultivation of *E. histolytica* has the advantage of continued exchange of a desired gas mixture and a higher ratio of culture medium on the membrane (1.75 ml.) to medium in the reservoir (70 ml.). Moreover, a number of cultures can be incubated simultaneously under controlled conditions (FIGURE 2) and, in our experience, the amoebae produced by this method are more easily handled in separation studies than those derived from standard tube cultures.

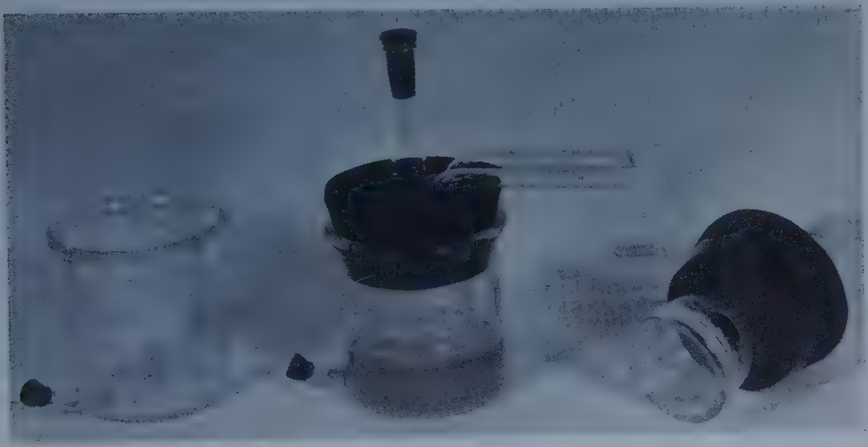

FIGURE 1. Assembly of a perfusion jar (PJ) for the cultivation of *E. histolytica*. Amoebae being cultured are introduced into the bell on the cellophane membrane through the tube of the bell. Gas phase is maintained through two tubes in the stopper and through two openings in top of the bell. Medium is introduced through the vaccine port.

In a long series of experiments summarized in TABLE 1, the original (Anfinsen *et al.*, 1946), and modified (Geiman, 1951) malaria culture media were adapted to obtain maximum growth and multiplication of *E. histolytica*. The different mixtures of nutrients tested gave results ranging from slight multiplication at 48 hours up to a maximum 29-fold increase in 24 hours. We must emphasize

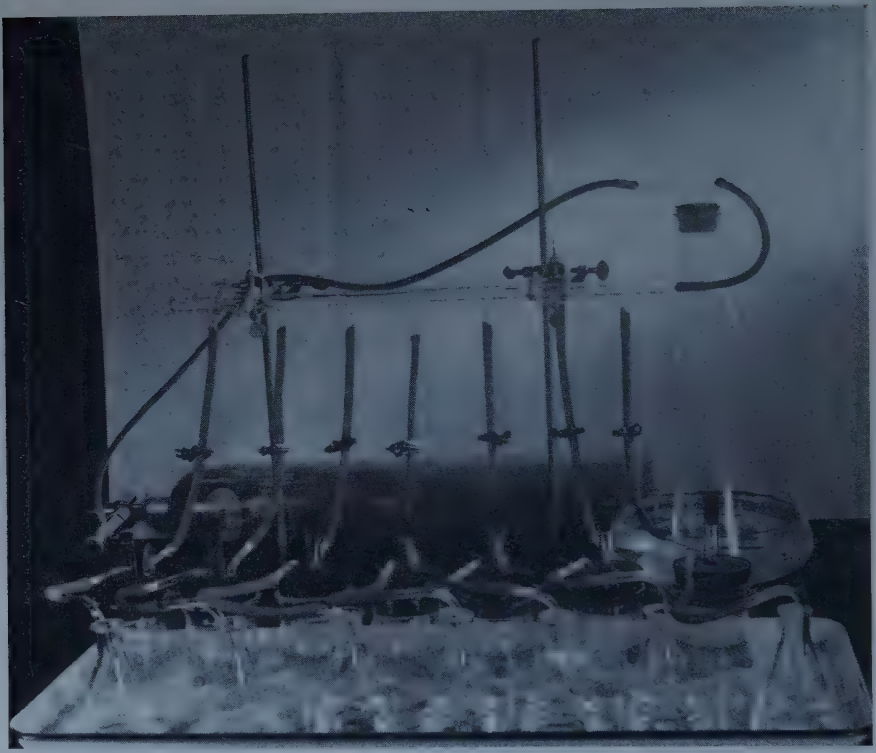

FIGURE 2. A photograph of seven PJ cultures of *E. histolytica* being incubated simultaneously using the same gas phase.

TABLE 1

PERFUSION JAR CULTURES WITH *E. histolytica* (HK-9 STRAIN)

No.	Medium	Gas Phase	Fold Increase	
			24 Hrs.	48 Hrs.
1	Medium for malarial parasites + serum + liver	95% N_2–5% CO_2		2.0
2	Same as (1) without $NaHCO_3$ but with phosphate buffer	99.7% N_2–0.3% O_2		8.4
3	Same as (2) without PABA, vitamins, amino acids, or purines and pyrimidines	"	16.6	
4	Same as (3)	99.83% N_2–0.17% H_2	23.5	
5	Same as (3) with 4x liver	"	28.8	

Adaptation of malaria culture media for the cultivation of *E. histolytica*. The composition of medium 5 is shown in TABLE 2.

that these studies were conducted with the HK-9 strain and with a mixed bacterial flora. With the HK-9 strain growing in the PJ vessel and medium shown in TABLE 2, we are able to obtain up to two million amoebae in 24 hours from a starting inoculum of approximately 65,000 amoebae. The culture medium and the mixed bacterial flora are not of known composition at present but at least the HK-9 strain gives reproducible results (TABLE 3). At the time the comparative experiments with four strains were done, the HI-1 strain had not become adapted to the medium in use.

TABLE 2
CULTURE MEDIUM[1, 2, 3]

(contains per liter)	
Na_2HPO_4	1.247 g.
KH_2PO_4	0.158 g.
KCl	0.366 g.
NaCl	5.200 g.
$MgCl_2 \cdot 6H_2O$	0.181 g.
$CaCl_2 \cdot 2H_2O$	0.066 g.
$NaC_2H_3O_2 \cdot 3H_2O$	0.926 g.
Glucose	2.586 g.
Ascorbic acid	0.062 g.
Liver concentrate (Lederle #3649-34)	0.15 ml.
Horse Serum	86.2 ml.

1. Initial pH set at 8.2.
2. Each P.A. jar contains 500 mg. of solid $CaCO_3$.
3. Gassed with 99.83% N_2 and 0.17% H_2.

Culture medium being used for the mass production of *E. histolytica* (HK-9 strain) for chemical analysis and metabolic studies.

TABLE 3
COMPARATIVE EXPERIMENTS*

$$\text{Fold increase} = \text{ratio of } \frac{\text{final harvest}}{\text{initial inoculum}}$$

Strain	Fold Increase		
	Expt. I	Expt. II	Average
HK-9	29.5	22.4	25.9
HK-14	39.5	16.2	27.8
DKB	26.2	13.7	19.9
HI-1	1.6	5.4	3.5

* The results of two experiments to compare the fold increase in 24 hours of four strains of *E. histolytica* in the PJ cultures and with the medium shown in FIGURE 4.

Growth curve. The growth curve shown in FIGURE 3 is of considerable interest because of the drastically different results shown between multiplication in the PJ vessels using a liver medium and the standard liver infusion agar (LIA) tubes. You will note the very short lag phase lasting about 6 hours and the rapid rate of multiplication during the next 18 hours, when compared with the prolonged lag phase in the LIA tubes. We believe that the superior results in the jar cultures are due, in large part, to the improved physiological environment at the time of inoculation and the maintenance of a constant gas phase.

For our chemical studies, we are interested in obtaining amoebae from cultures during the maximum period of exponential growth because we want amoebae in the best physiological state possible for chemical analysis and metabolic studies. We do not want amoebae harvested from cultures after the growth peak is passed and after degeneration sets in. Consequently, we are harvesting amoebae from PJ cultures at approximately 24 hours after inoculation.

In exploring the possibility of increasing the yield of amoebae from our cultures, we calculated the generation time of PJ cultures to know whether or not we are increasing the rate of multiplication. Using the formula (modified from Buchanan and Fulmer, 1928) shown in TABLE 4, the average generation time of HK-9 trophozoites grown in PJ cultures is about 4.9 hours. For comparative purposes, we have attempted to interpret and calculate generation times from growth curves given by Balamuth and Howard (1946) and from total yields obtained by Everritt (1950). Using different strains of amoebae and culture methods, the results in TABLE 5 show that the first group of workers obtained shorter generation times when much smaller inocula per unit volume of media were used. Everritt (1950) was not able to increase yields proportionately or decrease generation times by increasing the inoculum ten-fold—an inoculum that more nearly approaches the number of amoebae used to seed our perfusion jar cultures. Brackett and Bliznick (1947), using 15—5200 amoebae of the NIH 103 strain per tube and an incubation period of 3 to 6 days, obtained 2 to 2.5 divisions per day. Obviously, then, the PJ culture method

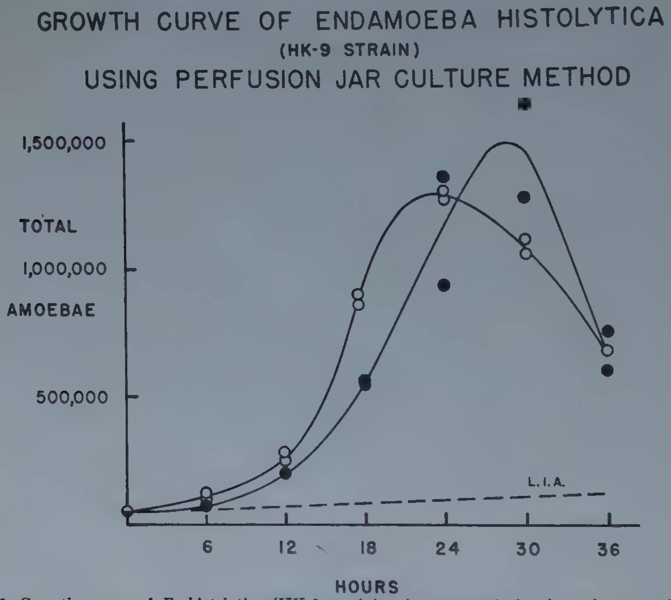

FIGURE 3. Growth curves of *E. histolytica* (HK-9 strain) using the perfusion jar culture method and liver infusion agar (L.I.A.) slants.

is giving a generation time with *E. histolytica* that is approaching the best results obtained by other investigators. We are interested, however, in obtaining at least one or two more generations per culture vessel either by adjusting the inoculum or by prolonging the exponential period of growth. At the present time, we are experimenting with the amount of nutrients in the reservoir on the theory that key substances are depleted from the medium at approximately 24 hours. The discovery of these essential or accessory substances might make it possible to further shorten the generation time and increase culture yields.

TABLE 4

GENERATION TIME

$$b = B\,2^n$$
$$\log b = \log B + n \log 2$$
$$n = \frac{\log b - \log B}{\log 2}$$
$$n = 3.3 \log \frac{b}{B}$$

$$\frac{b}{B} = 30$$
$$n = 3.3 \log 30$$
$$n = 4.9 \text{ generations}$$
$$g = \frac{t}{n} = \frac{24}{4.9} = 4.9 \text{ hrs.}$$

b = Final 24 Hour Results
B = Initial Inoculum
n = Number of Generations
g = Generation Time
t = Time in Hours

* Method of calculating the generation time of *E. histolytica* growing *in vitro*, adapted from Buchanan and Fulmer, 1928.

TABLE 5

In vitro GENERATION TIME OF *E. histolytica*

Reference	Strain	Medium	Inoculum (No. per mm.^{3})	Yield at 24 Hours (No. per mm.^{3})	Generations	Gen. Time Hours
Balamuth and Howard (1946)	NRS	Eggyolk infusion	5	984	7.6	3.2
		Eggyolk infusion and liver	12.3	10,414	9.7	2.5
		Cleveland and Collier	12.3	6,150	8.9	2.7
				(at 48 hours)		
Everitt (1950)	22	Eggyolk infusion	5	810	7.3	6.6
		Balamuth (1946)	50	930	4.2	11.5
	23	Eggyolk infusion	5	2,420	8.8	5.5
		Balamuth (1946)	50	2,120	5.4	8.9

Metabolism. To quote Gunsalus (1951): " . . . the factors controlling growth are subject to chemical laws and, however complex, are understandable and interpretable in terms of the chemical processes involved." Obviously, our knowledge about the metabolism of the cystic, metacystic, and trophozoite stages of *E. histolytica* is nowhere near being interpreted in this fashion. To unravel and arrive at an understanding of the chemical composition and biochemical behavior of the organism, controlled cultivation of the parasite is necessary to supply quantities of the amoebae that will give reproducible data. This is the basic reason for proposing the standardization of strains and methods of cultivation before we can obtain valid metabolic data for comparison with the results of other investigators.

Scattered metabolic data for *E. histolytica* are available (see reviews previously quoted) on the oxidation-reduction potentials required for growth, pH and temperature range for survival and multiplication, the requirement of cholesterol, carbohydrates, and complex mixtures of vitamins, and the possible production of proteolytic enzymes. This information obtained with different strains under a wide variety of *in vitro* conditions is too difficult to compare in the time available here. In any case, our knowledge is too scant to outline the metabolic pattern with any degree of certainty.

In conclusion, we wish to state that the methods outlined above are being used to produce amoebae for analysis of their chemical composition and subsequent metabolic studies. There is considerable hope that the gaps in our knowledge about *E. histolytica* will be filled in the not-too-distant future. Many investigators are at work, and the lines are converging to a solution of the biochemical and pathogenic nature of the dysentery amoeba of man.

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THE CULTIVATION OF *DIENTAMOEBA FRAGILIS*

By Leon Jacobs
Federal Security Agency, Public Health Service, National Institutes of Health, National Microbiological Institute, Bethesda, Md.*

Observations on the cultivation of *Dientamoeba fragilis* have been made by several investigators. Cultures have been used in survey work and in morphological studies. Thomson and Robertson (1925) probably were the first to cultivate this protozoon using the medium of Boeck and Drbohlav (1925). Their crude cultures were contaminated with *Blastocystis* and were not maintained long. Cleveland and Sanders (1930) made some incidental observations on *Dientamoeba* during the course of studies on *Endamoeba histolytica.* They reported that the liver infusion agar medium which they developed was practically specific for *E. histolytica* and that *D. fragilis* failed to grow in it. Cleveland and Collier (1930) isolated *D. fragilis* only once in six trials at culturing this organism in the liver infusion medium, but they found that the use of Loeffler's dehydrated beef serum slants provided for easy isolation and abundant growth. Dobell, in 1940, summarized the results of his years of study of *D. fragilis* and stated that this protozoon is "one of the easiest of the human intestinal amoebae to isolate and grow *in vitro.*" He used inspissated horse serum or egg, with an overlay of dilute egg white or serum-Ringer's, and a supplement of rice as his media. *D. fragilis*, he reported, feeds voraciously on bacteria and starch grains and also ingests red blood cells under some circumstances. Brug (1936, 1936a) used Dobell's medium of a coagulated horse serum slant with a serum Ringer's overlay and rice, and obtained some interesting results in his manipulations of these cultures. He observed a lag phase of the amoebae in fresh medium and attributed it to the size of the inoculum or the amount of old medium or bacteria introduced with the amoebae.

D. fragilis has been considered as a pathogen by a number of workers, but certainly no definite proof of its pathogenicity has yet been obtained (Wenrich, 1944). Furthermore, the method of transmission of this parasite is still unknown. With the idea that standardized culture material might serve as a convenient tool for further studies with this protozoon, some efforts have been made in this Laboratory to obtain cultures of *D. fragilis* with single species of bacteria, and also without any associated organisms. These attempts will be summarized briefly here.

A crude culture of *D. fragilis* (strain 400) was given us in November 1950 by Miss Lucy V. Reardon of this Laboratory. It had been established by her from a human stool specimen and maintained in the Locke's-egg-rice medium from February 1950. Miss Reardon also furnished another culture (400-NRS) into which the NRS bacteria had been introduced following treatment with antibiotics. Both cultures showed fair growth with about 5 or less amoebae per lower field, in examinations of sediment.

The technique used for trials at establishing *D. fragilis* with single species of bacteria consisted mainly of experiments with antibiotics. Since the manipulations involved in studies of this sort are an essential part of the history of the cultures, it is best to combine the descriptions of the various media and methods used along with the presentation of the results obtained.

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* Laboratory of Tropical Diseases.

It was known, from the observations of Dobell and Brug, that *D. fragilis* feeds on bacteria and also is dependent on the size of the bacterial population. No previous attempts, however, had been made to determine whether or not it was necessary that the bacteria required for the growth of this protozoon be alive. Also, the studies of Dobell and Wenrich indicated that *D. fragilis* was considerably different morphologically from the other intestinal amoebae. Therefore, since there was no reason to assume for *D. fragilis* the same dependence on viable associates that exists for *E. histolytica*, the very first tests performed here were designed to observe the growth of *D. fragilis* with dead bacteria.

For this purpose, the same type of medium used by this author in obtaining minimal growth of *E. histolytica* without viable bacteria (Jacobs, 1947) was employed. L.E. medium was conditioned by a 24- or 48-hour growth of *Escherichia coli*, then heated at 60 to 65 degrees C. for one hour, and provided with rice. Penicillin and streptomycin, in final concentrations of 200 to 400 units and 4 mg. per milliliter respectively, were then added, and the medium was inoculated with the sediment from crude cultures. In no case, was growth obtained in such cultures, unless there was also evidence of the proliferation of bacteria or yeasts.

In a few additional tests, the saline overlay of the conditioned medium was replaced with 50 per cent chick embryo extract. These tubes also failed to show growth of *D. fragilis*, and the bacterial growth was not entirely eliminated by the antibiotics.

On the other hand, relatively poor growth of *D. fragilis* in the crude cultures was considerably improved by the addition of streptomycin or penicillin singly either in conditioned medium or fresh medium. As examples, two series of cultures can be described.

Series I was derived from one culture of heat-treated conditioned medium which was inoculated with strain 400-NRS and to which penicillin (200 units per ml.) was added. Good growth of the amoebae (5 to 10 amoebae per low power field) was obtained, and the culture was transplanted into additional tubes of identical medium every 3 or 4 days for a total of 6 transfers. After the sixth transplant, 4 cultures were treated with streptomycin for a total of 12 to 14 transplants at 3- or 4-day intervals. In general, the growth of amoebae in these tubes was excellent, with 10 to 20 or more amoebae visible per low power field, and it remained so after the use of antibiotics was discontinued. Thioglycollate broth tubes and Gram-stained smears revealed the presence of only one species of anaerobic bacteria in most of the cultures derived from this series.

Series II was similarly derived from the NRS culture of strain 400. Four tubes of heat-treated conditioned medium containing 10 mg. per ml. of streptomycin were the original cultures of this series. They were maintained for 15 transplants in streptomycin-containing fresh L.E.R. medium and, thereafter, were continued without antibiotics. Thioglycollate broth tubes and blood agar plates from these cultures revealed, in most of the tubes, the presence of only one form of anaerobic bacteria. The growth of the amoebae in this series was also generally as good as that in series I.

No attempt was made to identify the species of bacteria supporting growth of *D. fragilis* in these 2 series of cultures. Instead, since it was amply demonstrated that this protozoon could tolerate fairly large concentrations of antibiotics, efforts were made to substitute known bacterial species for the unknown species present in the crude cultures or in those derived from them.

The technique of substitution of bacteria in cultures of *E. histolytica* had previously been used successfully (Jacobs, 1950). It was possible to establish *E. histolytica* with 3 different bacterial species (4 strains) merely by inoculating these penicillin-resistant organisms into cultures of amoebae growing with the penicillin-sensitive *Streptococcus hemolyticus*. After a few transplants in penicillin-containing medium, the streptococcus was eliminated and the amoebae remained in culture with the new bacteria.

Obviously, because there were at least several species of bacteria in the crude cultures of *D. fragilis*, and because cultures like those in series I and II contained bacteria which had survived treatment with antibiotics, this simple technique could not be used here. A modification of the technique was developed, based on previous observations by the author (1947) and by Balamuth and Howard (1946) that the lag phase of *E. histolytica* could be shortened or eliminated by allowing growth of bacteria to proceed for 24 hours before introducing the amoebic inoculum.

The species of bacteria to be substituted for the original flora was cultivated for 24 or 48 hours in the medium prior to the inoculation of amoebae. Just before the amoebae were added from the crude cultures, a mixture of penicillin and streptomycin and, in some cases, sulfadiazine, was added. The cultures were then transplanted daily into the conditioned medium plus drug, for at least 7 days. The cultures were observed on several occasions during this interval for growth of amoebae. If growth was obtained and when a sufficient number of transplants had been made in the presence of the drugs, the cultures were transplanted first into the conditioned medium without antibiotics and then maintained in the usual L.E.R. medium.

By this means, cultures of *D. fragilis* have been established with *Clostridium perfringens*. The cultures were derived from the crude strain 400 and transplanted daily into 24-hour *Clostridum perfringens* in the Locke's-egg-rice medium containing also 200 units of penicillin, 2 mg. of streptomycin, and 0.1 mg. of sodium sulfadiazine per ml. of overlay. Excellent growth of amoebae was observed in these tubes, 24 hours after they were set up, and they continued to show such growth thereafter. They were transplanted 7 times at 24-hour intervals in the presence of antibiotic and sulfa into the conditioned medium, and then the conditioned medium was used for another 7 daily transplants without the drugs. Thereafter, they were maintained routinely with transplants into the fresh L.E.R. medium. Blood agar plates, thioglycollate broth tubes, and Gram-stained smears from this series have shown the presence of only *Cl. perfringens*.

This result has been repeated in similar tests done with cultures originating from series I described above, in which transplants were done at less frequent intervals and only streptomycin and penicillin were used. On the other hand, attempts at establishing *D. fragilis* in culture with *Aerobacter aerogenes*, *Alcaligenes faecalis*, *Staphylococcus aureus*, *S. albus*, and organism *t* by this method have thus far failed. The amoebae have failed to grow in the L.E.R. medium conditioned by these bacteria when inoculated from the crude cultures of strain 400 and treated with the drugs as described above. Some growth has been obtained with *Escherichia coli* in the presence of streptomycin and penicillin under similar conditions, but the cultures were not carried long enough to rule out the presence of more than one species of bacteria. Further work is planned with this technique and modifications of it.

The failure of *D. fragilis* to become established, indeed to grow with organism *t*, in cultures initiated by the substitution method, was surprising. It was felt that perhaps insufficient growth of organism *t* was obtained in the first 24 hours in the L.E.R. medium; so some tests were done with 48-hour conditioned medium, but these were also negative in outcome. As a further modification of the technique, the liver infusion agar medium was employed. First of all, it was found that the crude culture of strain 400 could be maintained in this medium without difficulty. Then the medium was conditioned by the growth of organism *t* for 48 hours and treated and inoculated as above. The only tube in which growth of amoebae was obtained by this method was contaminated with a *Staphylococcus*.

It is apparent that our results with the use of antibiotics on cultures of *Dientamoeba fragilis* have not been highly successful. It has been possible on a few occasions to obtain cultures of this protozoon with what appear to be single species of bacteria. It is possible that, had sufficient time for its use been available, the micro-isolation technique would have been more profitable. Although our ability to manipulate cultures with antibiotics has been limited by a number of factors, certain interesting results have been obtained, which will now be summarized.

First of all, *Dientamoeba fragilis* resembles *Endamoeba histolytica* and *Endamoeba coli* in that growth of this protozoon has been obtained only in the presence of viable associated organisms.

Secondly, when it has been possible to separate the protozoon from its original flora, *D. fragilis* has grown abundantly in the presence of anaerobic bacteria. The similarity to *E. histolytica*, which also grows best with anaerobic bacteria, is striking.

Third, the growth of *Dientamoeba fragilis* in liver infusion agar medium was easily accomplished.

Fourth, *D. fragilis* grows well during the first 24 hours in medium conditioned by the prior growth of certain species of bacteria, even when bactericidal or bacteriostatic drugs are also present. It tolerates fairly high concentrations of penicillin, streptomycin, and sulfadiazine. Advantage has been taken of these characteristics to devise a technique whereby the amoeba may be established with a chosen species of bacteria while its original flora is eliminated.

Fifth, considering the morphological differences which exist between *Dienta-*

moeba fragilis and *Endamoeba histolytica*, the similarities in growth requirements of these 2 protozoa appear surprisingly great.

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DISCUSSION OF PAPERS ON INTESTINAL AMOEBAE

By Charles W. Rees
Federal Security Agency, Public Health Service, National Institutes of Health, National Microbiological Institute, Bethesda, Maryland*

As was pointed out by Mr. Phillips, some significant progress has been made toward the goal of cultivating *Endamoeba histolytica* under conditions that may yield information on problems of physiology of the parasite, and on serodiagnosis, chemotherapy, and pathogenesis of amoebiasis. Also, as indicated by Phillips, only limited information has been obtainable from cultures of the amoeba with a flora comprising a number of species of bacteria. On the other hand, the amoeba does not pass through all stages of the life cycle in any of the cultures that have been developed during recent years with selected single species of microbial associates and, in this respect, are inferior to those produced by Dobell and others in which encystation may be brought about. A method of producing cysts *in vitro* under conditions permitting analysis of the various factors is urgently needed.

The usefulness of cultures of *E. histolytica—Trypanosoma cruzi*, as well as of their immediate fore-runners; the cultures of Shaffer and Frye, as sources of bacteria-free amoebae for inoculation into medium, and into experimental animals, has been well demonstrated. The establishment of new strains of the amoeba into such cultures, however, especially from stool specimens, requires the preliminary use of methods of isolation. In our experiences, the technique of microisolation has been proved to be of great value for such work, especially when used in conjunction with later methods involving the use of antibiotics. Antibiotics alone will eliminate many species of bacteria without demonstrable harm to the amoebae, but the method is time-consuming and is ineffective against some species of bacteria occurring in stools. The inability of a number of investigators to obtain significant information on the metabolism of the amoebae with the use of Phillips's or Shaffer-Frye cultures, points up the need for pure cultures. In this connection, it is gratifying to learn of Dr. Shaffer's progress in cultivating the amoeba with living cells of chick embryos in the absence of bacteria. Another milestone on the road to pure cultivation has thus been passed. While opinions have been expressed that the amoeba is entirely dependent on live symbionts, and that metabolic studies can be conducted on amoebae derived from such cultures, the complications are formidable. Continued efforts to achieve associate-free cultivation of the amoeba are, therefore, indicated.

The significance of the investigations of Geiman and Becker cannot be appraised on the basis of the material presented in their paper. The title indicates data on metabolism, but those presented were almost entirely on cultural procedures. The statement that *E. histolytica* does not require vitamins and certain other growth factors is not borne out by data obtained by us. In a medium prepared from egg white the amoeba failed to grow with organism *t* unless the medium was enriched with a vitamin-cholesterol formula.

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* Laboratory of Tropical Diseases.

The paper by Doctor Jacobs on *Dientamoeba fragilis* demonstrates the usefulness of his pioneer work on the effectiveness of antibiotics in eliminating species of bacteria from amoeba-cultures. He is familiar with both the micro-isolation technique and the use of antibiotics, and the choice of the latter method here was based on the fact that *D. fragilis* does not form cysts. His cultures with *Clostridium perfringens* as the only microbial associate may prove useful in ascertaining whether *D. fragilis* is pathogenic for experimental animals.

SOME EFFECTS OF HOST ENVIRONMENT ON THE COURSE OF LEISHMANIASIS IN THE HAMSTER*

By Leslie A. Stauber

Bureau of Biological Research, Rutgers University, State University of New Jersey

The host of a parasite is part or all of that parasite's habitat, ecological niche, or culture medium whichever way you wish to state it. It is obvious, therefore, that those factors which affect the suitability of a culture medium, both physical and chemical, such as temperature, pH, O_2 tension, and the presence or absence of growth factors and inhibitors, will affect the growth and development of the parasite *in vivo*. This is abundantly evident for the culture tube in the reports given at this conference.

Alterations in the host's physiology may change the nature of the host as a culture medium. That many vertebrate hosts have excellent homoiostatic mechanisms which protect them (and thus protect their parasites also) against such changes merely makes the experiments more difficult to perform and to interpret.

Ever since the days of Pasteur, Metchnikoff, and their students it has been recognized that host body temperature is an important factor in infectious processes and, depending on the particular host and parasite chosen for study, alterations of the body temperature of the host may affect the course of infection of the parasite in that host. Thus, the high body temperature of birds is inimical to the development of anthrax in them. Pasteur, Joubert, and Chamberland,[1] however, found that infection of the hen was made possible by plunging the hen in cold water. The work of Gibier[2] with anthrax in the frog is just the opposite. When cultures of these bacteria were introduced into the dorsal lymph sac of frogs held at 37°C the frogs died with the bacteremia which developed. At room and refrigerator temperatures, the frogs were able to destroy the anthrax bacilli and survive. In neither of these cases was the surviving host deficient as a culture medium, or habitat, except in so far as its body temperature deviated far enough from that of the optimum for the development of the parasite so that the host defenses were successful in ridding it of the parasite. This is all the more true since Dieudonné[3] showed further that by selecting strains of anthrax capable of growing abundantly at 12°C he was able to kill frogs held at the lower environmental temperatures previously shown to favor survival of the frog.

In host-parasite relations it is not always as easy as in the above cases to separate host-stimulatory from parasite-inhibiting aspects. Fever, so common in infectious disease, has been claimed beneficial to the host in that the phagocytic activity of the leucocytes and the other defense reactions are enhanced. The practical application of this in artificial fever therapy is well known.

More obscure effects of environmental conditions on the host may also be reflected in the behavior of the parasites. Many of the avian malarias are highly synchronous in their periodic asexual reproductive cycle. L. G. Tali-

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aferro[4] refrigerated erythrocytic stages of synchronous *Plasmodium cathemerium* for 12 hours and found that when they were returned to a canary the length of the asexual cycle was shortened for several cycles so that in a few days the time of segmentation of the parasites was back to normal. G. H. Boyd[5] showed further that, by imposing an artificial 28-hour day on the canary, the length of the asexual cycle could be lengthened to 28 hours accordingly. He[6] also determined that, on placing the canary under continuous illumination, the synchronism of parasite reproduction was soon almost completely disrupted and that shifting the normal hours of light and darkness to other time periods caused shifts in the time of segmentation of the parasites. These observations have been abundantly confirmed.

The most logical interpretation of these various findings is that the host's activity and rest cycle is responsible for the diurnal rhythms observed in these animals or that one of the rhythms controls the host's activity and rest periods.[7] Canaries have a striking diurnal temperature rhythm[8] which, like the normal environment, is higher during the hours of light than during the hours of darkness of each day. Some years ago, we showed[9] that exposure of the infected canary to unusual environmental temperatures could disturb the parasite's rhythm either in its synchronicity or by causing a shift in the time of segmentation of the plasmodia. The data suggested further that one stage of the parasite, the young trophozoite in this case, was more susceptible to host influences. That some stage is susceptible to control must be inferred from the very existence of such a noticeable yet labile synchronous cycle of reproduction.

The amplitude of the oscillations of such host conditions as body temperature, slight or great as the case may be, may considerably influence the behavior of a parasite in a host. Huff[8] noted as much as a 6°C range in canaries in a 24-hour period and upon awakening a rise as rapid as 0.12°C per minute. It is important to note that the mean values for body temperature (or any of the many other physiological conditions which show diurnal rhythms[10]) may not be significantly altered by the altered environmental conditions imposed by the experimenter. If one of the extremes of amplitude, however, is at a critical level for a particular process a small deviation here may have a proportionately greater influence on the process. Take, for example, the loss of synchronism in the malaria parasite when the canary is kept continuously in the light.[6, 9] It is likely that such a canary feeding and sleeping at numerous shorter intervals (a polyphasic type of behavior[10]) shows a smaller amplitude of change in the body temperature than the monophasic canary on a schedule of 12 continuous hours of light and 12 of darkness. The mean body temperature could be essentially unchanged but the extremes might be decreased in amplitude to a degree which would alter the asexual reproductive cycle of the parasite.

Several workers have reported strains of avian malaria poor or lacking in gametocytes.[11, 12] Associated with this was general lack of synchronism of the asexual cycle. Caldwell[13] obtained his gametocyteless strains by *in vitro* exposure of parasites to temperatures lethal to most of the organisms. Other observations[14] show that one of the gametocyteless strains arose during a summer heat wave in Chicago when even uninfected birds were dying in the animal rooms. It seems almost beyond question, then, that profound effects on a parasite may be produced by unusual environmental conditions surrounding the host.

The very interesting findings of Thompson and Winder[15] showed that the saurian parasite, *Plasmodium floridense*, grows more rapidly and reaches its peak much sooner in the lizard, *Anolis carolinensis*, when kept at 30°C than when kept at 20°C. This temperature effect was not caused by an increase in the number of merozoites per segmenter but rather by a shortening of the asexual cycle. However, these environmental temperatures are within the physiological range for the lizard and host resistance, both innate and acquired, was apparently speeded proportionately with parasite growth rate. No effects like those indicated above for the avian malarias were observed. It should be noted here that Hegner[16] reported earlier that exposure of canaries infected with *P. cathemerium* to unusual environmental temperatures and humidities (100°F and 50 per cent humidity) produced more severe infections with prolonged patent periods. But no special effects on the parasite cycle were mentioned as occurring in these experiments.

The leishmaniae are considered by most workers to be primarily parasites of the insect gut and only secondarily in their evolutionary history have they become established in the vertebrate. In several respects, they act as though they were poorly adjusted to the vertebrate hosts. Although *Leishmania donovani* is regularly fatal to the hamster, in our experience, even with very large inocula the course of the infection is slow in development, requiring two to three months for a fatal consequence even in the generalized infection. In culture,[17, 18, 19] in the chick[20, 21, 22] and duck embryos[23] and in the insect gut,[24] growth of the organism is more abundant at temperatures below those of its mammalian hosts. The optimal range for all three species of *Leishmania* in the yolk sac of the chick embryo lies between 23° and 28°C[21] where it kills 6- to 9-day old embryos in 3 to 5 days. Growth is less abundant on either side of this range. In tissue cultures,[25, 26] at 37°C, L-d bodies occur in the cells or tissue present and leptomonads in the fluid of the medium but again the leptomonads apparently do not grow as abundantly as at lower temperatures, although Weinman[26] found that *L. tropica* "tolerates body temperature for long periods in a culture medium containing cells".

The very recent report of Trager[27] furnishes some additional evidence. He showed that intermediate non-motile round or ovoid forms of *L. donovani* develop from the typical L-d bodies and will persist for more than twice as long as culture leptomonads when put in a medium containing an extract of human erythrocytes, human serum and nutrient broth at 37°C. He also found that the intermediate forms did not grow out from the L-d bodies if rabbit or guinea pig serum replaced the human serum or if nicotinamide was added in the presence of human serum. On the other hand, although leptomonads also quickly died in the presence of nicotinamide or rabbit serum at 37°C neither of these materials had more than a delaying effect on the leptomonads at 28°C. Finally, Senekjie[28] reported a thermal death time of 15–30 minutes at 40°C for leptomonads of all three species of *Leishmania*. These findings may mean that the altered physiology of the L-d body in the intracellular climate helps it avoid at least some of the consequences of the higher ambient temperature, though it emphasizes the differential effects of temperature on the various morphological stages of the *Leishmania.*

TABLE 1

THE EFFECTS OF EXPOSURE OF *Leishmania donovani*-INFECTED HAMSTERS TO REFRIGERATOR, ROOM OR INCUBATOR TEMPERATURES

Temp. of Exposure	Days Exposed		No. of animals at time of death	Spleen Wt. (mg.)	Av. Spleen Wt.-Body Wt. Ratio*	Av. "total" Lds.†
	In on Day	Out on Day				
			Experiment 1			
5–6°C	0	45–47	4	200–843	7.3	1,520
20–25°C	0	43–47	4	120–1,070	9.2	3,500
34–35°C	10	45–47	2	62 & 64	1.5	None
			Experiment 2			
20–25°C	0	74–77	3	510–830	9.6	10,450
34–35°C	35	42 & 77	2	65 & 80	1.0	None
			Experiment 3			
20–25°C	0	42	1	448	6.1	6,850
	0	54	3	324–561	7.5	5,400
34–35°C	14	42	1	175	2.8	350
	42	54	1	350	4.0	1,470
			Experiment 4			
20–25°C	0	46	2	231–360	6.1	7,400
	0	57	2	699–782	7.3	8,680
34–35°C	19	46	2	298–544	5.7	1,750
	47	57	2	497–766	10.4	14,250
			Experiment 5			
20–25°C	0	28	2	91–102	1.2	14
	0	42	4	174–465	4.1	861
34–35°C	14	28	2	118–173	2.0	177
	28	42	2	213 & 233	3.1	832
			Experiment 6			
20–25°C	0	28	2	218 & 346	3.5	195
	0	42	4	63–564	4.1	608
34–35°C	14	28	2	248 & 321	3.8	815
	28	42	2	372 & 478	5.5	2,350

* The Spleen Weight — Body Weight Ratio is expressed as milligrams of spleen per gram of body weight.

† 'Total Lds' is obtained by multiplying the *Leishmania* per spleen cell nucleus by the milligrams of spleen. The figure obtained is not the absolute value. The actual total number of *Leishmania* in the whole spleen is undetermined but many times the figure obtained.

On these grounds, we decided to place infected hamsters in the refrigerator at 5°C and in the incubator at 35°C and compare the course of infection with other animals kept at room temperature. The hamsters were infected by intraperitoneal inoculation of ground spleen suspension from heavily infected hamsters. The Khartoum strain of *Leishmania donovani* was employed. The extent of the infection was determined at necropsy from spleen impressions and recorded as leishmaniae per spleen cell nucleus.

The results of several experiments are shown in TABLE 1. The experiments presented show all the range of response from apparent cure (Experiments 1 and 2), to small decreases from the expected parasite count (Experiment 3 and the 46-day portion of Experiment 4), to no effect at all (57-day portion of Experiment 4 and Experiments 5 & 6). The table shows only small groups of animals. This is partly because of the available space in the incubator for running the experiments but it also indicates further selection of the data. There are two reasons for not including details on the other animals in these experiments. In most of the trials, especially Experiments 1, 2, & 7 (not in TABLE 1) high mortalities were observed in animals put in the incubator. Deaths usually occurred within 24 hours after exposure. Thus, there were 6 other animals placed at 34–35°C in Experiment 1, 8 others in Experiment 2, and 10 in Experiment 7. Furthermore, no animals dying from leishmaniasis or other causes, whose tissues were examined *post mortem*, are included in the above tabular material even when apparently confirmatory. This is especially true for animals in the incubator since *post mortem* changes of even a few hours at 34–35°C would make the findings unreliable. Therefore, only data from those animals sacrificed by asphyxiation, whose tissues were immediately sampled, are included in TABLE 1. When an effect was produced both absolute and relative spleen size as well as parasite density were decreased by the exposure of hamsters to the high environmental temperatures (Experiments 1, 2 & 3). Some emphasis should be placed on the apparent cures in Experiments 1 & 2 since, in over 2500 infections studied to date, only one other possibly uninfected animal was encountered. It is, therefore, highly unlikely that these apparent cures are due to errors in infecting the animals.

It is important to note that no implication is here made that the body temperature of the hamster has been altered and that this is the direct factor involved. While some observations on body temperature have been recorded, it is obviously necessary that experiments must be set up predictably leading to apparent cures before these will have much significance.

Attempts to duplicate this effect with the metabolic stimulator 2-4 dinitrophenol have failed, either when fed in the hamster diet at concentrations as high as 0.2 per cent or injected subcutaneously in doses as high as 1.5 mg. per day. The dosage ranges used in the several experiments included concentrations showing no apparent signs of drug toxicity in the animals on test.

Summary

Exposure of hamsters infected with *Leishmania donovani* to high environmental temperatures, 34–35°C, led to variable results in individual experiments but included two experiments with apparent cures in all animals so exposed and surviving to time of sacrifice. Much more work is necessary before the effect produced or the nature of the processes involved can be further delineated.

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FACTORS AFFECTING THE SUSCEPTIBILITY OF ERYTHROCYTES TO AN INTRACELLULAR PARASITE

By R. Barclay McGhee

Laboratories of The Rockefeller Institute for Medical Research, New York, N. Y.

Following injection of washed erythrocytes of various species of animals into the blood stream of developing chicken embryos which were infected with the avian malaria parasite, *Plasmodium lophurae*, certain of these erythrocytes became parasitized.[1, 2] Of the 4 species of animals whose cells were subject to invasion, 3 were rodents (the mouse, baby rat and rabbit), while the remaining animal, the pig, belonged to the order *Artiodactyla.* The red blood cells of other members of these orders, such as the hamster and guinea pig among the rodents, and the sheep and cow among the artiodactyls, were not subject to parasitization. Two intact animals, the baby mouse[3] and baby rat,[4] were infected, proving that the cell susceptibility was not an artifact engendered by pre-treatment or conditioning by the embryo.

Similarities in susceptibility of erythrocytes of species widely divergent in their taxonomic status and the dissimilarities of cells from animals as closely related as the adult rat, with its insusceptible cells, and the baby rat, whose erythrocytes are susceptible, prompted attempts to find additional similarities in respect to morphology and composition. Morphologically, there are vast differences. The bird erythrocyte is nucleated and relatively large with the long axis in the neighborhood of 14 microns, as contrasted to the anucleated blood cell of the mouse with a diameter of about 5 microns. Basically, however, the structure of mammalian erythrocytes consists of an envelope with or without an inner matrix, the stromata, and hemoglobin. Present beliefs are that the hemin portion of the hemoglobin is the same regardless of species, while the globin may not be as different in various animals as crystallization would lead one to believe.[5] Chemical analyses of the entire erythrocyte served to classify animals into 2 groups: those with high potassium—low sodium concentrations, and those with high sodium—low potassium.[6] All susceptible erythrocytes fell in the category of high potassium—low sodium. Classification of erythrocytes in respect to their organic acid-soluble phosphorus content further divided the high potassium group (TABLE 1).[7] It is notable that susceptible erythrocytes were in a group having the greatest concentration of organic acid-soluble phosphorus. The concentrations of the adenosinetriphosphate portion of the acid-soluble phosphorus were not in harmony, but the readings for diphosphoglycerate fractions were quite similar. This latter fraction is not, however, present in birds, where it is replaced by phytic acid. The increased phosphorus concentration in the baby rat erythrocyte extended the similarities in regard to susceptible erythrocytes but was offset by the almost identical readings in baby guinea pigs. Whether the ATP or diphosphoglycerate fractions are similar has not yet been determined.

Having found that various species of erythrocytes, freed of their plasma and distinguishable from the erythrocytes of the embryo, were susceptible to invasion by the merozoites of *P. lophurae*, attempts were made to determine biologically, if possible, the reason for their susceptibility. To conduct any work along this line, it was necesary to use a cell more stable in the embryo circulation than that of the baby rat, pig, and rabbit and more susceptible than that of the mouse. The erythrocytes of the adult duck met these requirements and could be distinguished from embryo red blood cells by the shape of the nucleus.[8] These cells were so subject to infection that, during a 4-hour period, the majority of free merozoites entered them rather than the chicken embryo cells. During the period of 4 hours, there was an ever increasing number of parasites in duck erythrocytes while the parasitemia in chick cells remained relatively constant. Estimation of the numbers of available merozoites at the time of injection of the duck cells and their disposition at the end of 4 hours indicated that an average of 16 per cent entered duck cells with almost none entering those of the chick.

TABLE 1

THE RELATIONSHIP BETWEEN POTASSIUM-SODIUM AND PHOSPHORUS CONCENTRATIONS IN THE ERYTHROCYTE AND THE SUSCEPTILIBITY TO INFECTION WITH *P. lophurae*

Animal	Susceptibility	mM per 1000 grams†		Mg. per 100 cc.‡		
		Potassium	Sodium	Organic acid-soluble phosphorus	Diphosphoglycerate	ATP
Chick	+	97.3	7.1	96–95.2	67.0*	9–11
Pig	+	100.0	7.5	85.8	43.7	20–22
Rabbit	+	99.0	16.0	87.7	45.3	21.1
Mouse	+	100.0	7.5	85.8	51.8	12–16
Baby rat	+	—	—	87.0**	—	—
Adult rat	−	100.5	11.9	66.8	34.1	14.4
Baby guinea pig	−	—	—	82.0**	—	—
Adult guinea pig	−	105.0	15.0	59.7	30.1	10–11
Man	−	100–109	11.0	55.0	28.0	13.5

* Phytic acid.
† Kerr, 1937.
‡ Rapaport and Guest, 1941.
** My own determinations.

The fact that the majority of merozoites entered the introduced duck erythrocyte posed certain questions which may eventually throw light on the phenomenon of cell invasion. The duck erythrocytes never exceeded 30 per cent of the total cells present in the circulating blood stream of the embryo. The merozoite is not, so far as we know, possessed of any power of motility. The infection of any erythrocyte, therefore, must depend on a chance meeting of the merozoite and the erythrocyte. Theoretically, the merozoite would hit 3 chick cells to every 1 duck erythrocyte. Therefore, the greater invasion of duck erythrocytes would indicate that the surface of the duck cell is many times more receptive to the merozoite than that of the chick.

In light of these possibilities, it was decided to attempt to alter the surface or the interior of the duck erythrocyte to a point that further invasion by the parasites would be curtailed. By so doing, it might be possible to determine whether the stromata or hemoglobin or both are necessary for parasite penetration. The following experiments represent preliminary steps to resolve the questions outlined above.

Erythrocytes from adult ducks were washed twice, resuspended in isotonic saline, and mixed with cholera vibrio culture filtrate (a procedure designed to remove receptors from the cell surface[9]). Two tenths ml. of these erythrocytes were injected into infected embryos. Erythrocytes not exposed to the filtrate were used as controls. There was no difference in the percentage of merozoites entering the two batches of erythrocytes.

Erythrocytes were treated with steapsin to remove one of the hydrocarbon chains from the surface,[10] and injected as before. There was no change from the usual rate of merozoite penetration.

Duck erythrocytes were washed and exposed to carbon monoxide for 10 minutes, then injected into infected embryos. Merozoites entered these cells at a rate comparable to untreated erythrocytes.

Ghost cells were prepared using the method of Parpart.[11] Completely lysed cells were quickly removed from the embryo circulation. Partially hemolyzed erythrocytes, however, were not so quickly removed and could be found in substantial numbers even after 24 hours. These erythrocytes retained their original shape to some extent and had varying amounts of hemoglobin in them, generally in the form of bands radiating from the nucleus or around the periphery of the cell. The amount of invasion of these cells was sharply reduced although it was still possible to find parasites in partially hemolyzed erythrocytes. It is notable that all the parasites were located in areas where hemoglobin was present, and had produced pigment after 24 hours.

Duck red blood cells were exposed to varying concentrations of rabbit anti-duck red cell antiserum. The amounts of antibody finally chosen were less than 1 unit, because of intravascular agglutination. The amount of invasion of these erythrocytes was one-tenth of those of the control erythrocytes.

Of the various methods tried, the use of antibody and the partial lysis of the erythrocyte reduced susceptibility. Although these methods in themselves do not clarify the mechanism of susceptibility, they are promising enough to merit further work along similar lines.

In summary, it may be stated that erythrocytes exhibit graded degrees of susceptibility. In so far as is known, there are decided similarities in chemical composition among those cells subject to invasion. Preliminary investigations to determine the role of hemoglobin or stromata in the infection of the erythrocyte are not as yet conclusive, but methods of attack have been found which may, in the future, shed more light on the phenomenon of parasitization.

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FURTHER STUDIES ON THE EXTRACELLULAR CULTIVATION OF AN AVIAN MALARIA PARASITE

By William Trager
Laboratories of the Rockefeller Institute for Medical Research, New York, N. Y.

Since intracellular parasites have not been observed to develop extracellularly in culture media, it has been generally assumed that they must have special environmental requirements readily satisfied only by intact, living host cells.[4] How much of the intactness of the host cell is required, whether its physical structure, its labile metabolic intermediates, its enzymes or its coenzymes, or all of these factors, is not known, and may well be expected to be different for the different groups of intracellular parasites. The problem has been approached in recent years by studies on the respiratory metabolism *in vitro* of surviving suspensions of intracellular parasites freed from their host cells. It has been found that such suspensions of malaria parasites have all the enzymes of the glycolytic series and of the citric acid cycle[1, 9] possessed by mammalian tissues. Similarly, suspensions of rickettsiae appear to oxidize glutamate via the citric acid cycle.[2, 13] The respiration of suspensions of murine leprosy bacilli[5] was not stimulated by any of a variety of known substances of importance in cellular metabolism, but it was stimulated by bovine albumin and by yeast or liver extract. These fundamental studies have not as yet given any indication as to the peculiar nutritional or other requirements which might help to account for the obligate intracellular parasitism of the organisms concerned.

A somewhat different approach to the problem has been made in experiments with an avian malaria parasite, *Plasmodium lophurae*.[11, 12] Here, the organisms were freed from their host cells directly into a medium designed to support their relatively long survival. The extent of survival in the presence of various nutritional supplements and under different physical conditions was then followed by morphological methods. These studies have shown that the erythrocytic stages of *P. lophurae* survive and develop extracellularly in a concentrated extract of duck erythrocytes. The survival and development are favored by a number of metabolites of known chemical nature, especially by adenosinetriphosphate, pyruvate, malate, and coenzyme A. Since adenosinetriphosphate and coenzyme A are labile compounds which occur in nature chiefly within living cells, since both are of great importance in biosynthesis,[6] and since malaria parasites evidently require an external source of these compounds, the intracellular mode of life of the parasites becomes more understandable.

Under the best conditions which have so far been tested, *P. lophurae* survives and develops extracellularly *in vitro* at 40°C. for a period of 3 days. On the third day, only 5 to 10 per cent of the extracellular parasites seen in stained films appear degenerate,[12] but this proportion increases considerably by the fourth day. Obviously, some important requirements of the parasites are not met by the *in vitro* conditions so far tried. Nevertheless, these conditions support sufficiently good development of the organisms to permit more detailed exploration of their nutritional requirements. In the present paper, some experiments will be presented which illustrate the effects of certain changes in the culture medium and which emphasize the importance of the supplements previously shown to exert favorable effects.

Methods

Since these have been described in detail in the previous papers,[11, 12] they will be only briefly reviewed.

Erythrocytes from a duckling heavily infected with *P. lophurae* were suspended in duck erythrocyte extract, and subjected to the action of an anti-duck erythrocyte serum prepared in a rabbit, and guinea pig complement. In this way, most of the duck erythrocytes were hemolyzed and many parasites were completely freed from their host cells. For most experiments, 0.3 ml. of the 10 per cent suspension of hemolyzed erythrocytes and parasites was inoculated to each experimental flask containing 3.1 ml. of medium to be tested. The flasks were equipped with gas inlet and outlet tubes and received a slow current of a mixture of 95 per cent air and 5 per cent CO_2. They were rocked gently in an incubator at a temperature of 40°C. During the first 18 hours of incubation, nearly all the red cell remains and the parasites accumulated, as a result of the rocking, in a scum on the walls of the flask. This made it possible to draw off the culture fluid and replace it with fresh medium with a minimum of disturbance to the parasites. The culture fluid was changed after the first 18 hours and thereafter at 12-hour intervals. Recently, the formation of the scum has been insured and improved by first lining the lower portion of the wall of the flask with a thin layer of clotted normal duck plasma.[12]

During the course of the experiment, small samples of the scum were taken once daily with a fine pipette and used to prepare wet mounts and dry films. The wet mounts were examined immediately with a phase contrast microscope and the general condition of the free parasites was noted. The dried films were stained with Giemsa's stain and were used for counts of degenerate parasites and of the numbers of free parasites of normal appearance in different stages of development.

When living malaria parasites freed from their host cells are examined with the dark contrast phase microscope, their cytoplasm presents a characteristic, delicate, even, medium-bright appearance. Small deviations from this appearance can be readily seen. Parasites beginning to be abnormal may have cytoplasm either darker or brighter than that which is so characteristic of the parasites when they are freshly freed from their host cells or when they have remained in good condition after maintenance *in vitro*. Definitely degenerate parasites show cytoplasm that is dark, blotchy, or of an opaque brightness. All of these appearances have been figured in photomicrographs already published. The appearance of the parasites in the fresh preparations has been, in general, a more sensitive indicator of the degree of suitability of the cultural conditions than has been their appearance in stained films. The stained films, however, provide indispensable additional criteria, as well as a permanent record.

Red cell extract, the diluent used for its preparation, and the solutions of various supplements have all been prepared exactly as already described[11, 12] except where otherwise noted for particular experiments.

Results and Discussion

The source of carbohydrate. For these experiments, the red cell extract was prepared with the standard diluent containing glucose, or with a diluent in which the glucose was replaced by an equal weight of maltose, sucrose or glycogen. In the diluent with glycogen, the concentration of NaCl and KCl was increased by 10 per cent to compensate for the loss in osmotic pressure produced by replacing glucose with glycogen. The completed red cell extract was supplemented with coenzyme I and adenosine-3 phosphoric acid, but not with malate. Each flask received initially and with each change of culture medium the supplement of adenosinetriphosphate and pyruvate, but not coenzyme A concentrate. No difference was observed over a 3-day period in either the fresh preparations or the stained films from flasks containing glucose, maltose, or glycogen. Fresh preparations from flasks with sucrose showed by the second day larger numbers of dark and blotchy parasites than did preparations from control flasks with glucose. This result was also apparent in the stained slides (TABLE 1) which showed 2 or 3 times as many degenerate parasites in the sucrose flasks as in the glucose ones.

TABLE 1

THE EFFECT OF THE CARBOHYDRATE SOURCE ON THE EXTRACELLULAR DEVELOPMENT OF *P. lophurae* AS SEEN IN STAINED FILMS

200 parasites in successive microscopic fields were counted and classified.

Preparation	Time *in vitro* (days)	Medium*	No. of parasites of normal appearance		No. of degenerate parasites
			2–4 nuclei	Total	
Original	0	—	13	197	3
Flask 1	1	Glucose	24	200	0
2			31	199	1
3		Maltose	33	199	1
4			30	199	1
5		Sucrose	18	199	1
6			27	198	2
Flask 1	2	Glucose	34	189	11
2			28	190	10
3		Maltose	31	188	12
4			30	191	9
5		Sucrose	24	174	26
6			16	162	38

* Red cell extract prepared in the usual manner with a diluent containing the indicated carbohydrate. The media were supplemented with coenzyme I, adenosine-3-phosphoric acid, adenosinetriphosphate and pyruvate.

P. lophurae maintained intracellularly in suspensions of duck erythrocytes has been found previously to survive as well in a medium with glycogen as in a medium with glucose.[10] Respiration of malaria parasites has been observed to be stimulated by maltose as well as by glucose, but not by sucrose (or a variety of other carbohydrates).[3, 8]

Glutathione and pyruvate. Glutathione was early found to prolong the survival of *P. lophurae* in suspensions of host red cells maintained *in vitro*.[7, 10] For this reason, and also because it occurs naturally in red cells at relatively high concentrations, it has been regularly included in the nutrient solutions used to make erythrocyte extract. In several experiments with extracellular parasites, the survival of the parasites was not as good as was to be expected with the media being used. It had been noted that the glutathione (from a bottle opened some time previously and kept in a refrigerator) used for these experiments gave a turbid solution. A direct comparison of medium prepared with this sample of glutathione with medium prepared with a fresh sample of glutathione showed that the latter supported better extracellular survival than the former. The actual chemical difference between the two samples was not determined. In all subsequent work, only glutathione from small bottles opened for not over three weeks and stored cold was used.

TABLE 2

THE EFFECT OF ADENOSINETRIPHOSPHATE AND SODIUM PYRUVATE IN THE PRESENCE OF MALATE ON THE EXTRACELLULAR DEVELOPMENT OF *P. lophurae* AS SEEN IN STAINED FILMS

200 parasites in successive microscopic fields were counted and classified.

Preparation	Time *in vitro* (days)	Medium*	No. of parasites of normal appearance		No. of degenerate parasites
			2–4 nuclei	Total	
Original	0	—	5	199	1
Flask 1	1	No further supplement	4	199	1
2			3	193	7
3		Supplemented with ATP and pyruvate (Prep. A)	7	200	0
4			15	200	0
5		Supplemented with ATP and pyruvate (Prep. B)	12	200	0
6			12	198	2
Flask 1	2	No further supplement	20	187	13
2			12	194	6
3		Supplemented with ATP and pyruvate (Prep. A)	18	196	4
4			13	196	4
5		Supplemented with ATP and pyruvate (Prep. B)	19	198	2
6			11	197	3
Flask 1	3	No further supplement	15	164	36
2			17	167	33
3		Supplemented with ATP and pyruvate (Prep. A)	19	179	21
4			25	178	22
5		Supplemented with ATP and pyruvate (Prep. B)	18	177	23
6			12	182	18

* The medium was red cell extract prepared in the usual manner and supplemented with coenzyme I, adenosine-3-phosphoric acid and malate. Further supplements were added where indicated.

A somewhat similar situation was noted with a commercial sample of sodium pyruvate which did not support as good survival as was regularly obtained with several preparations of sodium pyruvate made from two different batches of pyruvic acid.[11] When a new preparation of sodium pyruvate was made, it was tested against an old one, as in the experiment of TABLE 2. This experiment also shows that the favorable effect of the supplement of adenosinetriphosphate and sodium pyruvate (at final concentrations of approximately 2 and 5 millimols respectively, per liter) is evident in the presence of malate, a fact not previously demonstrated. It is interesting to note that the development of parasites with 2 to 4 nuclei was delayed in the flasks without the supplement of adenosinetriphosphate and pyruvate (TABLE 2, Day 1, Flasks 1, 2, as compared to Flasks 3–6). In the flasks without the supplement, more degenerate parasites were apparent on days 1, 2 and 3 than in the flasks with the supplement but, even in the former, the proportion of degenerate parasites was not so great as had been generally found in earlier experiments, where the medium also lacked malate.[11]

TABLE 3

THE EFFECT OF DILUTION OF THE RED CELL EXTRACT ON THE EXTRACELLULAR DEVELOPMENT OF *P. lophurae* AS SEEN IN STAINED FILMS

200 parasites in successive microscopic fields were counted and classified.

Preparation	Time *in vitro* (days)	Medium*	No. of parasites of normal appearance		No. of degenerate parasites
			2–4 nuclei	Total	
Original	0	—	3	200	0
Flask 1	2	Full strength	27	198	2
2			21	200	0
3		¾ strength	28	193	7
4		½ strength	25	190	10
5		Second extraction	29	190	10
6			17	192	8
Flask 1	3	Full strength	24	191	9
2			35	192	8
3		¾ strength	30	190	10
4		½ strength	27	185	15
5		Second extraction	29	179	21
6			21	186	14

* The medium was red cell extract prepared as described in the text. Dilutions were made with the standard diluent and adjusted to pH 6.8. All the media were fully supplemented with coenzyme I, adenosine-3-phosphoric acid, malate, adenosinetriphosphate, pyruvate, and a coenzyme A concentrate.

Effect of dilution of the red cell extract. The usual erythrocyte extract, designated as full strength extract, was the dark red supernatant solution obtained after mixing one volume of frozen-thawed duck erythrocytes with one and one-third volumes of nutrient solution and centrifuging for 50 to 60 minutes in a horizontal centrifuge at 3000 RPM. The further dilution of this extract with nutrient solution was accompanied by poorer survival of extracellular *P. lophurae*. Since the nutrient solution with gelatin has a pH of 5.9, whereas the full strength extract prepared with it has usually a pH of 6.8, it was necessary to adjust the pH of the dilutions. In the experiment the results of which are shown in TABLE 3, the pH of each dilution when freshly prepared was adjusted to that of the full strength extract.[6, 8] The medium designated as "second extraction" was made by resuspending the sediment from the first centrifugation in an equal volume of nutrient solution and again centrifuging for 50 minutes. The pH of the supernatant was adjusted to 6.8. It will be seen (TABLE 3) that, while dilution of the red cell extract gave progressively poorer survival, the number of degenerate parasites even in the "½ strength" and "2nd extraction" media was not large. It is to be noted that these flasks were all fully supplemented with adenosinetriphosphate, pyruvate, malate and a coenzyme A concentrate (Armour's "liver coenzyme concentrate" added initially and with each change of medium to supply a final concentration of about 6 Lipmann units per ml.). When the supplements of adenosinetriphosphate, pyruvate and coenzyme A were omitted from a flask with ½ strength red cell extract, very much worse survival of the extracellular parasites was obtained (TABLE 4, Flask 6, as compared with Flasks 3, 4). A comparison of fresh preparations made on the second day from Flasks 1 and 2, 3, and 4, and 6 showed in a striking way the effects of the media on the free parasites. The parasites from Flasks 1 and 2 mostly had even cytoplasm of a medium brightness. Those from Flasks 3 and 4 had a similar appearance except that their cytoplasm was distinctly darker, while in those from Flask 6 the majority had cytoplasm of a very dark, dense appearance. When red cell extract was omitted from the medium (but introduced at 0 time with the inoculum of para-

TABLE 4

THE EFFECT OF DILUTION OF THE RED CELL EXTRACT AND OMISSION OF CERTAIN SUPPLEMENTS ON THE EXTRACELLULAR DEVELOPMENT OF *P. lophurae* AS SEEN IN STAINED FILMS

200 parasites in successive microscopic fields were counted and classified.

Preparation	Time *in vitro* (days)	Medium*	No. of parasites of normal appearance		No. of degenerate parasites
			2-4 nuclei	Total	
Original	0	—	3	200	0
Flask 1	1	Full strength red cell extract	11	199	1
2			12	200	0
3		½ strength red cell extract	10	198	2
4			13	198	2
5		No red cell extract	7	188	12
6		½ strength, with ATP, pyruvate and coenzyme A omitted	5	193	7
Flask 1	2	Full strength	15	193	7
2			14	188	12
3		½ strength	40	180	20
4			33	169	31
5		No red cell extract	4	30	170
6		½ strength, with ATP, pyruvate and coenzyme A omitted	9	122	78

* The standard diluent (see text) was adjusted to pH 6.8 and used to make red cell extract in the usual manner except that the pH of the final extract was brought back to 6.8 with 0.6 NHCl. The media were fully supplemented (see footnote, TABLE 3) unless otherwise indicated.

sites and hemolyzed cells at a concentration of about 10 per cent of the full strength concentration), and all the supplements were used, the survival of the free parasites was almost as good at one day as in the half-strength extract without supplements (TABLE 4). By the second day, however, few normal parasites remained in the medium without red cell extract. The development of parasites with 2 to 4 nuclei was as negligible in the unsupplemented half-strength red cell extract as in the medium without red cell extract (TABLE 4).

Summary

The effects of certain constituents of the culture medium on the survival and development *in vitro* of *Plasmodium lophurae* freed from their host erythrocytes have been studied. During a period of 3 days, the parasites survived as well in media with maltose or glycogen in place of glucose as in the medium with glucose. The survival was considerably worse, however, in a medium with the glucose replaced by sucrose. Media prepared with an old preparation of glutathione or a commercial sample of sodium pyruvate did not support as good survival of the parasites as did media containing glutathione from recently opened bottles and pyruvate prepared in the laboratory from pyruvic acid. Dilution of the erythrocyte extract gave progressively poorer survival. With half-strength red cell extract, however, supplemented with adenosinetriphosphate, pyruvate and coenzyme A, the proportion of degenerate parasites on the 2nd and 3rd days was not great. It was much increased if these supplements were omitted.

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STUDIES OF THE INFLUENCE OF 2,6-DIAMINOPURINE UPON THE CONTENT OF KAPPA IN *PARAMECIUM AURELIA*, VARIETY 4*

By C. Chester Stock, Mary Williamson, and Winifred E. Jacobson
Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, New York, N. Y.

Many compounds, such as purines and pyrimidines, have been tested for their ability to restrain growth of neoplasms in animals.[1-7] These studies are based upon an assumption of a difference between normal and cancer cells in their patterns of metabolism of nucleic acid precursors. It is further assumed that the difference is sufficiently great to induce selective damage in the abnormal cells by use of appropriate anti-metabolites. This approach to cancer chemotherapy would be equally applicable whether the neoplastic process is due to a virus or a mutated genic structure for both are dependent for their function upon the component nucleic acids. Thus far, few of the compounds tested as potential antimetabolites of nucleic acids have exerted a high degree of growth restraint upon any type of neoplasm *in vivo* and none has been curative. In tissue culture, a substantial degree of selective injury to some neoplastic cells has been demonstrable.

As interference with nucleic acid metabolism is attractive on theoretical grounds, every effort to provide evidence of its practical possibilities seems indicated. Consideration of various biological systems revealed that the characteristics of the cytoplasmic factor kappa in the ciliated protozoan *P. aurelia*, as elucidated so beautifully by Sonneborn and his associates, would make it an ideal system for study.

Certain stocks of *P. aurelia* known as killers contain a sufficient concentration of kappa particles to produce paramecin,[8, 9] a substance lethal to other stocks of paramecia which lack kappa and are known as sensitives. A killer must always contain the cytoplasmic factor, kappa, and the killer (K) gene; kappa is gene dependent but not gene initiated.[10, 11, 12] Kappa is comparable in size to rickettsiae and some viruses. It contains desoxyribonucleoprotein and is self-duplicating at rates which may differ from the fission rate of the paramecia. Hundreds to over a thousand of these entities may be found in the cytoplasm of a single killer animal.[13, 14, 15] It was believed that if kappa could be inactivated or destroyed without injury to the host there would be evidence of a high degree of specificity of possible nucleic acid antagonism.

It was decided, therefore, to test various substances, chiefly purines and pyrimidines, for their ability to decrease kappa in paramecia without killing the paramecia or interfering with their ability to undergo normal fission subsequently.

The technique involved exposure of the paramecia to the test chemical under physiological conditions with subsequent determinations of the effect on kappa by use of the killer-sensitive test.[16, 17] *P. aurelia*, variety 4, stock 51 killer, mating type VII, was used. It was cultured in Sonneborn's baked lettuce

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medium inoculated with *Aerobacter aerogenes*. Ten to twenty paramecia grown at two fissions per day[13] for three or four days prior to exposure were exposed in 0.2 ml. of various dilutions of test chemical at pH 6.4–7.0 for 24 hours at 27°C. The salt solution described by Taylor and Van Wagtendonk for *Colpoda duodenaria* was used for making all dilutions.[18] Triplicate tests were run. After the incubation, the paramecia were examined for locomotion, general morphology, growth and reproduction. Then, single animals were isolated from the three highest dose levels into fresh culture and allowed to reproduce at maximum rate to render sensitive any in which kappa had been decreased appreciably as a result of the exposure to test chemical. The resultant clones after the rapid growth were tested in the killer-sensitive test.

TABLE 1

BIOLOGICAL ACTIVITIES OF 2,6-DIAMINOPURINE

- *L. casei* growth inhibition in defined medium[19]
- Inhibition "growth of estrogen stimulated chick oviduct[20]
- Interference in vaccinia virus production in tissue culture[21]
- Benefits in mouse leukemia[22]
- Bone marrow hypoplasia, megaloblastosis, etc. in rats and dogs[23]
- Bone marrow hypoplasia, etc. in swine[24]
- Chromosome abnormalities in mouse[25]
- Differential toxicity to normal and cancer cells of rat and mouse[26]
- Interference with Russian encephalitis virus production in tissue culture[27]
- Limited mouse protection from Russian encephalitis virus[28]
- Chromosome abnormalities in tissue culture[29]

TABLE 2

TESTS AGAINST KILLING ACTION OF *P. Aurelia*

Compound	Tolerated Level γ/ml.	Effect on *P. Aurelia*
2,6-Diaminopurine	1000	Inhibits killing 100 γ+
Adenine	500	Increased growth
Adenosine	1500*	Increased growth
Adenylic acid (yeast A and B)	1500	Increased growth
Adenylic acid (muscle 5-PO$_4$)	1500	Increased growth
9-α-D-arabofuranosidyladenine	25	None
9-α L-arabofuranosidyladenine	1500	None

* 1500 γ, highest level tested.

Chosen for test first was 2,6-diaminopurine* which previously had shown effects in various biological systems. These are listed in TABLE 1. It was found that 2,6-diaminopurine could decrease the effective amount of kappa[30] and was in fact the only one of nearly 70 purines and pyrimidines tested which showed this capacity.[31] The marked specificity of the effect is illustrated by the lack of action from 8-aza-adenine and from various substituted 2,6-diaminopurines.[31] It was of interest to note the difference in toxicity of the two arabofuranosyl adenines; though they were without apparent effect upon kappa, there obviously is a physiological action indicated by the marked difference in toxicities of the D and L forms (TABLE 2).

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* The 2,6-diaminopurine and numerous other compounds were kindly supplied by Doctor George H. Hitchings, Wellcome Research Laboratories, Tuckahoe, N. Y.

A few antibiotics were found effective (TABLE 3). Several are in confirmation of earlier work on streptomycin[16] and chloromycetin.[32] It was necessary to determine that the action of 2,6-diaminopurine and the antibiotics was not through interference with growth of the bacteria and thereby affecting their usefulness as nutrient for the paramecia. It was shown that there was no decrease in the number of bacteria produced.[31]

When an antimetabolite action is suspected, it is natural to test various materials and particularly the natural metabolite for ability to block the action of the antimetabolite. A few materials were studied for their ability to protect kappa from the adverse effect of 2,6-diaminopurine. It was found (TABLE 4) that adenine, adenosine and the adenylic acids were effective, decreasing in the order named. The action of the samples of desoxy- and ribonucleic acids possibly could be due to their polynucleotide adenine content or to the presence of small amounts of impurities though the quantities effective do not suggest the latter.

TABLE 3

TESTS AGAINST KILLING ACTION OF *P. Aurelia*

Compound	Tolerated Level* γ/ml.	Effect on *P. Aurelia*
Penicillin G	1500	None
Streptomycin	1500	Inhibits killing 50 γ^+
Dihydrostreptomycin	1500	Inhibits killing 25 γ^+
Aureomycin	25	Inhibits killing 10 γ^+
Terramycin	200	Inhibits killing 100 γ^+
Chloromycetin	500	Inhibits killing 200 γ^+
Neomycin	1500	May slow growth

* 1500 γ highest level tested.

TABLE 4

BLOCKING OF 2,6-DIAMINOPURINE EFFECT ON KILLER ACTION

Compound	Molar Ratio Compound/2,6-Diaminopurine for Blocking
Adenine	1:100
Adenosine	1:30
Adenylic acid (yeast)	1:5 or 10
Adenylic acid (muscle)	1:15 or 25
Guanylic acid	No effect at 2/1
Desoxyribonucleic acid	200 γ:500 γ*
Ribonucleic acid	1000 γ:500 γ*

* Weight ratio.

As the means had been provided by the work of others, we studied the quantitative aspects of the action of 2,6-diaminopurine and its blocking by adenine, adenosine, and adenylic acid. For this, the expansion or high temperature technique of Sonneborn was used.[9, 16, 33] This is a method for estimating the amount of kappa present by growing the paramecia under conditions in which the total amount of kappa remains essentially constant and is diluted among the dividing paramecia until there is approximately one kappa per paramecium.

Then, based upon the number of sensitives to viable clones and the number of fissions and the Poisson law of distribution,* the number of kappa per paramecium after exposure to test chemicals may be calculated.[33, 34]

By this procedure, we find that the amount of kappa present in the 2,6-diaminopurine exposed animals is approximately 2 per cent that of the saline controls[35] (TABLE 5). In TABLE 6 is shown the blocking experiment in which the corresponding reduction of kappa from 2,6-diaminopurine is to 3 per cent. When adenosine is present with 2,6-diaminopurine the percentage change in kappa is 425 that of the control. It is to be noted that this is more than that present with adenosine alone.

TABLE 5

REDUCTION IN KAPPA FROM 2,6-DIAMINOPURINE (100 γ/ml) MEASURED BY EXPANSION TECHNIQUE

Pre-expansion Treatment	No. Fissions at 33.8°	No. of Isolates	Sensitives / Viables	Kappa at Start of Expansion	
				Calc. No.	% of Control
None, initial control	9	45	22/42	330	160
	9	45	25/44		
	9	45	22/45		
2,6-Diaminopurine 100 γ/ml. for 24 Hours	7	60	58/60	4	2
	7	60	59/60		
	7	60	58/60		
Salt solution Culture fluid 24 Hour control	9	45	27/43	205	100
	9	45	25/42		
	9	45	31/39		

TABLE 6

BLOCKING OF 2,6-DIAMINOPURINE EFFECT ON KAPPA MEASURED BY EXPANSION TECHNIQUE

24 Hour Treatment No.	No. Fissions at 33.8°	No. of Isolates	Sensitives / Viables	Kappa at Start of Expansion	
				Calc. No.	% of Control
None, initial control	9	45	15/44	565	90
2,6-Diaminopurine 500 γ/ml.	4	15	4/14	19	3
Adenosine 500 γ/ml.	11	30	10/29	2270	360
2,6-Diaminopurine + Adenosine 500 γ/ml. each	10	30	2/28	2700	425
Salt solution Culture fluid	11	30	22/30	635	100

Thus, we have shown a more or less selective interference in nucleoprotein metabolism. These results were obtained at dose levels with an insignificant number of deaths and retention of ability to divide subsequently when placed in new medium. Some abnormal forms, however, were produced. It is not certain how the 2,6-diaminopurine is acting. It has no strong chemical reactivity to achieve its effect like the nitrogen mustards. It presumably acts to prevent the utilization of adenine in the formation of additional kappa. This concept would appear supported by staining procedures showing fewer stained kappa particles[35] after exposure to 2,6-diaminopurine. It is possible that an abnormal kappa with unusual purine content is produced. Whatever the mechanism of the decrease in kappa content, we believe we have demonstrated a more or less selective interference with nucleic acid metabolism under physiological conditions.

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* For this application of the Poisson distribution law, $Po = e^{-m}$
Po = proportion of animals with no kappa (sensitives at end of expansion).
m = mean number of kappa particles per animal at end of expansion.
e = base of natural logarithm.

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GIANT INDIVIDUALS AND VIGOR OF POPULATIONS IN TOKOPHRYA INFUSIONUM*

By Maria A. Rudzinska†

Department of Biology, Washington Square College, New York University, New York, N. Y.

One of the peculiar features of *Tokophrya infusionum* is the ability to feed without interruption.[1] If a *Tokophrya* feeds heavily and constantly for over 48 hours, it changes to a giant individual exceeding, on the average, 120 times the previous size of the body. Giants are dark, opaque with short tentacles or without tentacles. Their life span is greatly shortened; their reproduction rate greatly reduced and finally they cease to reproduce at all. These observations led to the supposition that the amount of food might influence the life span of normal individuals in *Tokophrya*. A detailed study was performed to determine the effect of heavy feeding contrasted with meager diet and intermittent starvation.[2, 3] Since *Tokophrya* feeds only on living ciliates which become attached to its sucking tentacles it was easy to regulate the amount of food and time of feeding. Using a special method,[3] it was possible to keep individual records on many *Tokophrya* throughout their life span in the culture tube (screw-capped). It was found in these experiments that individuals kept on a meager diet with intermittent starvation live, on the average, twice as long as those fed abundantly every day. The experiments confirmed the expectation that heavy feeding introduced daily is responsible for shortening the life span in normal individuals and giants. It seems probable that, when large amounts of food are incorporated daily, a state of auto-intoxication is induced. Additional food material is being brought in while the old has not yet been digested. A situation is met within which the *Tokophrya* is overloaded with food material. A part of this ingested food is removed into the embryo during reproduction. When heavy feeding is going on daily, however, the rate of reproduction drops.[1] It might be also that the *Tokophrya* is unable to remove adequately the accumulating waste products, because of unfavorable factors taking place in connection with heavy feeding, such as the change of the shape of the body and condensation in bulk of food ingested,[3] and also because of the drop of the reproduction rate. Many investigators (Calkins,[4] Kidder,[5] Diller,[6] Kudo[7]) suppose that, during reproduction, waste materials are eliminated from the nucleus. A lower reproduction rate would favor an accumulation of waste materials. It seems probable that, under these circumstances, auto-intoxication takes place. This would explain the shortening of life span in overfed individuals and giants.

Since giants or individuals approaching this state are characterized by a very low reproduction rate and a very shortened life span it was supposed that the number of giants may be in close relation to the vigor of populations. This was found to be true in recent experiments. A comparative study was made of three lines of populations all derived from the same strain which I received through the courtesy of Doctor Lilly, and fed on *Tetrahymena geleii* generously

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† Present address: Rockefeller Institute for Medical Research, New York, N. Y.

supplied by Doctor R. P. Hall. One of the lines (A), was kept about one year under normal conditions. "Normal conditions" mean room temperature 18°C–26°C; transfers made once or twice weekly. Line B was about 8 months under normal conditions and then placed in the refrigerator at 4°C for a period of 6 weeks; when it was removed from the refrigerator and transferred to normal conditions. Line C was kept for 5 months on a starvation diet. Food was introduced once in two weeks and lasted for only one day on the average.[8, 9] From such a starved culture, kept in the same culture tube for a period of 5

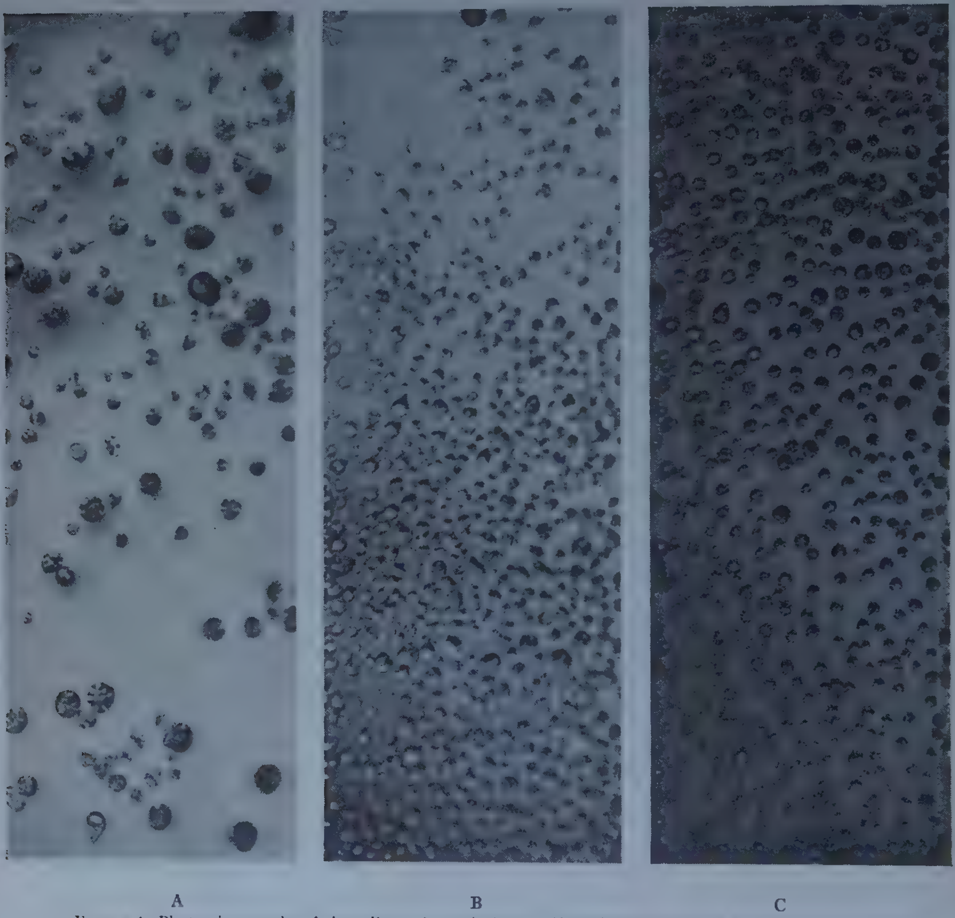

FIGURE 1. Photomicrographs of three lines of populations of *Tokophrya infusionum* deriving from the same strain. 48 ×.

A. Population kept under normal conditions for one year. Note many giant individuals and the loose and scattered distribution of animals.

B. Population kept for 8 months under normal conditions and thereafter for 6 weeks at low temperature +4°C. Note that only a few individuals are approaching the size of giants; the distribution of organisms is dense.

C. Population kept on a starvation diet for 5 months and thereafter at low temperature +4°C for a period of 6 weeks. Note the even size of organisms and their even and dense distribution.

At the time the photograph was made, all three lines were for a period of 6 weeks under the same conditions as to the temperature, amount and kind of culture medium, amount and kind of food and frequency of transfers.

months, subcultures were made which, after one month in normal conditions, were transferred to the refrigerator at 4°C for 6 weeks, and afterwards transferred again to normal conditions. In all three lines, as culture medium, a filtered brewers' yeast solution as recommended by Lilly[10] was used. These three lines of populations have been now for a period of 8 weeks under the same conditions as to the amount and kind of food, amount and kind of medium, temperature and frequency of transfers. The difference between these three lines is striking as to the number of giants, number of individuals and reproduction rate. While there were 30–40 per cent of giants in population A, kept continuously under normal conditions; in population B, after refrigeration for six weeks, only about two per cent were giants; and much less in population C, which was at first kept on a starvation diet and then placed in the refrigerator. The respective ratios of reproduction are approximately 2 (A); 5 (B); 10 (C). The differences are illustrated in FIGURE 1. The three photographs, A, B, and C, each represent one of the above lines. A represents a population kept continuously under normal conditions, showing a large number of giants, while B and C indicate populations kept under unfavorable conditions for some time with only a few individuals approaching giant size. B and C are characterized by their density, uniform size of individuals, and uniform distribution. In A, on the other hand, the individuals are irregular in size and loosely scattered.

It is clear from these experiments that vigorous populations (B, C) have a small number of giants, a high reproduction rate (expressed in density), while a weak population has a large number of giants and a low reproduction rate (A). Lilly,[11] in his experiments on *Tokophrya* with purines and pyrimidines, obtained similar results. He found that the number of giants decreases by adding to the medium purines and pyrimidines. At the same time, the vigor of the culture increases.

In connection with the differences among the three above described lines, the question arises as to how to explain the diminishing vigor of a population kept throughout the whole time in good condition and the increased vigor of those kept for some time on a starvation diet and/or at low temperature.

The possible answer seems as follows. When a population is kept under unfavorable conditions (starvation or low temperature), all the weak individuals die and only the strongest most resistant, are left. This would mean that unfavorable conditions act as a selective factor. This elimination of weak individuals strengthens the vigor of the population. Therefore, it seems to be probable that unfavorable conditions introduced periodically are able to preserve an undiminished vigor of populations in *Tokophrya infusionum*. It might be that the same process of elimination of weak individuals is occurring in nature where seasons of favorable conditions are interrupted by periods of stress.

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STUDIES ON THE FLAGELLA OF ALGAE.
II. FORMATION OF FLAGELLA BY *CHLAMYDOMONAS* IN LIGHT AND DARKNESS

By Ralph A. Lewin

National Research Council of Canada, Maritime Regional Laboratory, Halifax, N. S., Canada

The flagella of *Chlamydomonas moewusii* Gerloff play an essential role in the mating reactions of this protist (Lewin, 1952[a]). In the course of experiments on the effect of light on mating (Lewin, 1952[b]), it was observed that the development of flagella was itself dependent on conditions of illumination. Under certain conditions, these organelles may be resorbed in darkness, and can be induced to redevelop under the action of light, or by the addition of water, as described below.

Methods. Agar slants of the following composition were inoculated from a cell suspension, and were illuminated under fluorescent lights (300 foot-candles) at 23°C. It has not proved possible to cultivate this species on any medium in the absence of light (Lewin, 1950). After subjecting such cultures to various periods of darkness and illumination, samples of cells were washed off the agar surface and fixed with 0.1 per cent osmic acid, stained with 0.1 per cent gentian violet, and the flagella length of a suitable number of cells (usually 25) measured with the use of an ocular micrometer.

Composition of medium.

$Ca(NO_3)_2 \cdot 4H_2O$	1.0	g. per l.
$MgSO_4 \cdot 7H_2O$	0.2	"
K_2HPO_4	0.2	"
Na acetate	1.0	"
Na_3 citrate $\cdot 2H_2O$	1.0	"
Trace elements (Fe, etc.)		
Agar	10.0	"

Observations. The cells form flagella when grown on the surface of agar media (Lewin, 1952[a]), and are immediately capable of swimming when water is added in either light or darkness. However, the average length of flagella of cells maintained on agar in darkness falls gradually over a period of a week from 10–12μ to 2μ or less. Starvation beyond this period begins to result in a dying-off of the cells.

When cells with shortened flagella are submerged in distilled water, in light or darkness, the flagella commence to elongate at once, and regain their original length and activity within 1–2 hours (FIGURE 1). This rate of growth, if continued in linear fashion, would be equivalent to only 2 mm. per week. Flagella formation is not affected by small amounts of glucose, but is delayed by concentrations in which temporary plasmolysis is followed by rapid recovery and is suppressed by hypertonic concentrations exceeding 10 per cent (FIGURE 2).

When cells are illuminated *without* the addition of water, elongation of flagella is induced similarly, though less rapidly than in liquid media (FIGURE 1). An appreciable effect can be observed after 20 minutes illumination at 500 f-c.,

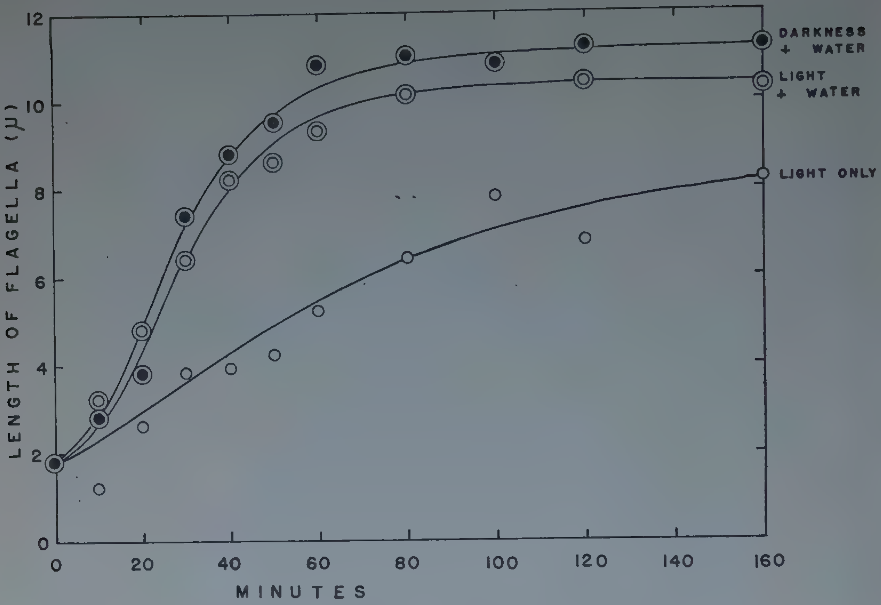

FIGURE 1. Re-formation of flagella in light and/or water.
(Cells grown in light 4 days: in darkness 5 days. Temperature: 23°C. Light: 450 f-c.)

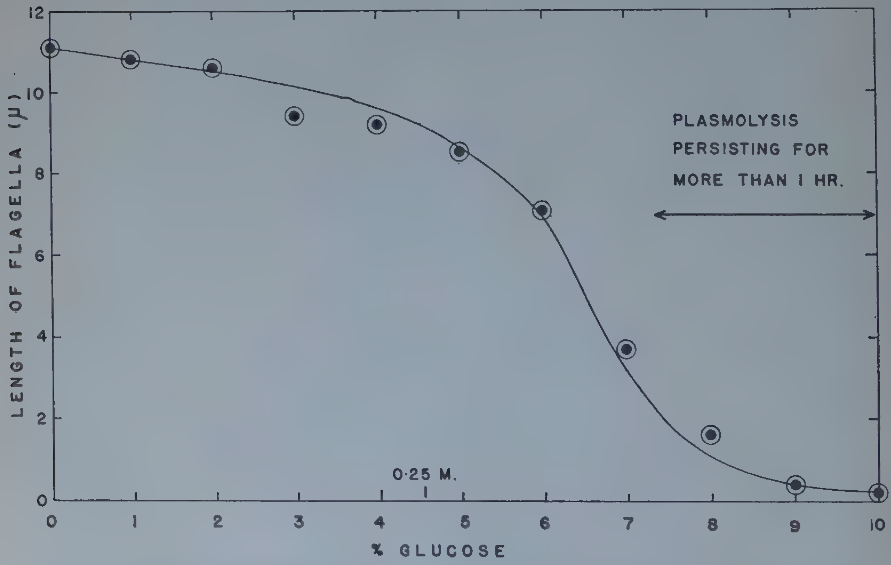

FIGURE 2. Re-formation of flagella in solutions of various osmotic pressure.
(Cells grown in light 4 days: in darkness 4 days. Temperature: 23°C. Light: il. Duration: 90 mins.)

or after 2 hours under as little as 5–10 f-c. There are preliminary indications that here, too, as in the activation of the mating process (Lewin, 1952^b), the action spectrum corresponds to that of chlorophyll, indicating that the energy for flagella re-formation is derived directly or indirectly from photosynthesis.

Discussion. The fact that re-formation of flagella can be induced not only in water, but also by the action of light, suggests that illumination may promote the uptake of water from the surrounding environment, a system analogous to that controlling stomatal movement in vascular plants. The development of flagella may perhaps follow the alleviation of incipient plasmolysis of cells on the agar surface. However, more than simple osmotic phenomena must be involved, since the formation of these organelles is a process of growth, presumably of protein synthesis, for which some endogenous source of energy must be mobilized when water is added.

Parenthetically, it should be pointed out that, in its ability to form flagella in water in darkness, *C. moewusii* appears to differ radically from *C. eugametos* (Moewus, 1943), although the two strains are morphologically similar and have been shown to be sexually compatible (Smith, 1951).

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